FACTORS AFFECTING THE DEVELOPMENTAL COMPETENCE OF PIG OOCYTES MATURED *IN VITRO*

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Abstract

Pre-pubertal pig oocytes possess lower developmental competence than those from adult pigs following *in vitro* maturation (IVM). Previous studies have demonstrated that exposure of pre-pubertal oocytes to 1 mM dibutyryl cAMP (dbcAMP), a membrane permeable cyclic adenosine monophosphate (cAMP) analogue, for the first 20 h of IVM improves the rate of blastocyst development. Developmental competence of *in vitro* matured pig oocytes has been reported to increase with increasing follicle size. In this thesis, experiments were carried out using pre-pubertal and adult pig oocytes to investigate the relationship between donor age, intra-oocyte cAMP level and follicle size in terms of oocyte maturation and developmental competence.

These experiments demonstrated that, while ovarian, follicular and oocyte morphology are immediately altered with the onset of puberty, pre-pubertal oocytes must be exposed to more than the first oestrous cycle to achieve improved developmental competence *in vitro*. Later experiments demonstrated that pre-pubertal oocytes accumulate less cAMP during IVM, undergo more rapid meiotic progression and display reduced rates of blastocyst development compared to *in vitro* matured adult oocytes. Treatment with dbcAMP for 22 h IVM increased the cAMP content of pre-pubertal oocytes, slowed meiotic progression during IVM and improved the rate of blastocyst formation. While the cAMP concentration of pre-pubertal oocytes was increased to levels similar to that of adult oocytes, rates of blastocyst formation remained lower, suggesting that additional factor(s) are required for oocyte maturation.

This thesis also examined the follicle size cohorts that make up the 3-8 mm aspiration range on pig ovaries. The surface of pre-pubertal ovaries contained around double the number of 3 mm follicles compared with adult ovaries. Blastocyst development of pre-

pubertal oocytes increased with increasing follicle size and was highest using oocytes from 5-8 mm follicles, while adult oocytes from all follicle size cohorts displayed similar high rates of blastocyst formation. The interaction between follicle size and cAMP content in pre-pubertal oocytes was examined next. Cumulus-oocyte complexes (COCs) from 3 mm follicles accumulated less intra-oocyte and inter-COC cAMP and displayed reduced cumulus expansion compared with COCs from 5-8 mm follicles. While dbcAMP treatment increased the cAMP content of oocytes from 3 mm follicles, it had no effect on the cAMP content of the whole COC. These findings suggest that inadequate levels of intra-oocyte cAMP during IVM contribute to the low developmental competence of pre-pubertal oocytes from 3 mm follicles, suggesting that cAMP transfer, production or degradation processes are incomplete. Analysis of steroid content from different follicle size cohorts revealed that the progesterone content of pre-pubertal follicular fluid (FF) increased with increasing follicle size, yet overall was lower than that of adults. This suggests that differences may exist in the gonadotropin-stimulated steroidogenic activity of granulosa cells of pre-pubertal COCs from different follicle sizes. Since progesterone secretion did not differ between pre-pubertal and adult COCs, it appears that the downstream pathway from the granulosa cell response rather than the actual quantity of progesterone is important for subsequent maturation processes.

These studies then examined gap junction communication (GJC) within the pre-pubertal COC during IVM to examine whether the positive effects of increasing follicle size and dbcAMP on intra-oocyte cAMP levels relates to improved cAMP transfer between the cumulus cell layer and oocyte. Cumulus cell-oocyte GJC during IVM was maintained for a longer period in pre-pubertal COCs from 3 mm follicles than in those from 5-8 mm follicles. Treatment with dbcAMP had minimal effect on GJC in either COC type,

thus the dbcAMP-induced increase in intra-oocyte cAMP levels appears independent of GJC. Differences in GJC during IVM together with the COCs ability to increase intra-oocyte cAMP levels during IVM, suggests that differences may exist in the quantity of gonadotropin receptors, which are responsible for cAMP production, within the cumulus layer of COCs from 3 mm compared with 5-8 mm follicles.

In conclusion, this thesis has demonstrated that an increase in intra-oocyte cAMP is necessary during maturation for completion and synchronisation of maturation and high developmental competence of the pig oocyte. Comparison of 3, 4 and 5-8 mm follicle sizes in the pre-pubertal pig, as described here, provides an excellent model for further investigation into the role of cAMP and the other factors required for co-ordination of oocyte nuclear and cytoplasmic maturation and subsequent embryo production.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except for when due reference has been made in the text.

I give consent to this copy of my thesis being made available in the University of Adelaide Library.

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Abbreviations

>	larger than
<	smaller than
+	plus
±	plus or minus
=	equals
5'-AMP	adenosine 5'-monophosphate
ana I	anaphase I
AREG	amphiregulin
ATP	adenosine triphosphate
BMP15/GDF9b	bone-morphogenic protein 15
BSA	bovine serum albumin
BTC	betacellulin
B-TCM	bicarbonate buffered-tissue culture medium
cAMP	cyclic adenosine monophosphate
^{o}C	temperature expressed as degrees celcius
CL	corpora lutea present on ovaries
COC	cumulus-oocyte complex
Cx	connexin
D I	diakinesis I
dbcAMP	dibutyryl cyclic adenosine monophosphate
DMAP	6-dimethylaminopurine
DMSO	dimethyl-sulphoxide
DNA	deoxyribonucleic acid
DO	denuded oocyte

E_2	17β-oestradiol
EGF	epidermal growth factor
ER	oestrogen receptor
EREG	epiregulin
FCS	fetal calf serum
FF	follicular fluid
FGF	fibroblast growth factor
fmol	femto moles
FI	fluorescence intensity
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
GDF-9	growth differentiation factor-9
GJC	gap junction communication
GV	germinal vesicle
GVBD	germinal vesicle breakdown
h	hour(s)
HB-GF	heparin-binding egf-like growth factor
hCG	human chorionic gonadotropin
H-TCM	hepes-buffered tissue culture medium
iAC	invasive adenylate cyclase
IBMX	3-isobutyl-1-methyxanthine
IGF	insulin growth factor
IGF-BP	insulin growth factor binding protein
IP(3)R	inositol 1,4,5-trisphosphate receptor
IU	international units

IVC	*in vitro* culture
IVF	*in vitro* fertilisation
IVM	*in vitro* maturation
IVP	*in vitro* production
KL/SCF	kit ligand/stem cell factor
LH	luteinising hormone
LHR	luteinising hormone receptor
MAPK	mitogen activated protein kinase
MGC	mural granulosa cell
MI	metaphase I
MII	metaphase II
min	minute(s)
mg	milligram(s)
mIU	milli international units
ml	millilitre(s)
µl	microliter(s)
mM	millimolar concentration
mm	millimetre(s)
µg	microgram(s)
µm	micrometre(s)
µM	micromolar concentration
MPF	maturation promoting factor
MPN	male pronucleus
mRNA	messenger ribonucleic acid
L	litre(s)

NCL	ovaries with no corpora lutea
NCSU	North Carolina State University
nmol	nanomoles
ng	nanogram(s)
P_4	progesterone
PB-NCSU	phosphate buffered North Carolina State University- 23 medium
PBS	phosphate buffered saline
PDE	phosphodiesterase
PI-3 kinase	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PR	progesterone receptor
PVA	polyvinyl alcohol
rhFSH	recombinant human FSH
RIA	radioimmunoassay
sec	second
SPM	sperm pre-incubation medium
TALP	tyrode-albumin-lactate-pyruvate
telo I	telophase I
TGFα	transforming growth factor α
TGFβ	transforming growth factor β
VEGF	vascular endothelial growth factor
vs.	versus

Chapter 1

Literature Review

1 Literature review

1.1 Introduction

The technological revolution in reproductive biology that started with artificial insemination and embryo transfer technologies during the 20th century has continued with oocyte *in vitro maturation* (IVM), *in vitro* fertilisation (IVF), parthenogenetic activation, *in vitro* embryo culture (IVC) and cloning of domestic animals by nuclear transfer from somatic cells, to name only a few. IVM has particular significance, being the platform technology for the abundant supply of mature, good quality oocytes for applications such as reducing the generation interval in important species, research to improve *in vitro* human reproduction and production of transgenic animals for cell therapies, protein production and for medical applications.

IVM exploits the large reserve of oocytes that exists in mammals from the time of birth, many of which are never ovulated and normally undergo atresia at various stages of follicular development in pre-pubertal or adult life (reviewed by (Wassarman 1988). While *in vivo* matured oocytes can be aspirated, the process is time consuming, expensive and yields less mature oocytes compared to IVM. In contrast, IVM utilizes immature oocytes that can be aspirated from follicles on the surface of ovaries obtained from the slaughterhouse when domestic species are processed for meat production. Despite the convenience of IVM, we still do not understand the precise factors and conditions occurring *in vivo* that yield the highest quality mature oocytes for positive fertilisation and embryo development outcomes, and hence we cannot completely imitate these conditions. Experimentally, IVM can be used as a tool to elucidate the requirements for optimal oocyte maturation.

In addition to its high agricultural value, the pig is an important domestic animal model for human medical and reproductive research (reviewed by Prather *et al.* 2003). Pig organs are similar to those of humans in terms of physiology and size, providing an excellent source of xenotransplantation organs. The timing of oocyte maturation is also similar between the pig and human, making pig IVM an ideal platform for development of human IVM technologies. Pig ovaries are readily available from the slaughterhouse, with large numbers of oocytes obtainable per ovary for IVM. Despite numerous improvements to pig *in vitro* embryo production (IVP) over the last decade, the efficiency remains inferior to both cattle and *in vivo* produced pig embryos (reviewed by Nagai 2001 and Prather *et al.* 2003). The low efficiency of pig embryo IVP appears mainly due to low rates of embryo development and fertilisation abnormalities including polyspermy and decreased male pronuclear formation (reviewed by Niwa 1993 and Nagai 1994). To advance both the medical and reproductive research that benefits from pig IVP, the IVM systems for pig oocyte maturation must be improved.

Oocyte maturation involves important nuclear and cytoplasmic modifications in the oocyte. Nuclear maturation refers to the changes that occur during the resumption of meiosis to ensure a haploid complement of chromosomes results from the previously diploid state. Cytoplasmic maturation refers to the changes that are essential for successful fertilisation and embryo development. Incomplete cytoplasmic maturation of the oocyte appears to account for the majority of problems with subsequent fertilisation and embryo development. *In vivo*, the pre-ovulatory luteinising hormone (LH) surge induces meiotic resumption of oocytes within selected follicles, while the physical removal of oocytes from their follicular environment for IVM induces spontaneous meiotic resumption (Pincus and Enzmann 1935). Variations in nuclear morphology in oocytes pooled from 3-8 mm porcine antral follicles results in a heterogenous

population of oocytes being utilized for IVP of pig embryos (McGaughey and Polge 1972; Nagai *et al.* 1997; Funahashi *et al.* 1997b). During IVM this heterogeneity results in cohorts of oocytes that reach metaphase II (MII) earlier than others, and age for the remainder of IVM (Grupen *et al.* 1997; Nagai *et al.* 1997; Funahashi *et al.* 1997b).

Poor embryo IVP efficiencies have been attributed to the use of pre-pubertal donors as an oocyte source, since the oocytes of pre-pubertal domestic livestock species display reduced rates of blastocyst development compared with those of adults (reviewed by Armstrong 2001). Pigs are generally slaughtered prior to reaching puberty, making pre-pubertal pig oocytes the most abundant for IVM and related reproductive technologies (O'Brien *et al.* 2000; Marchal *et al.* 2001; Grupen *et al.* 2003; Ikeda and Takahashi 2003; Sherrer *et al.* 2004; Bagg *et al.* 2006). In Australia, pre-pubertal pigs constitute the majority of pigs slaughtered for meat production and hence make up the majority of ovaries available for IVM. Donor age aside, maturational and developmental ability is closely correlated with oocyte size and follicle diameter in a number of species including the pig (reviewed by Abeydeera 2002). Since the antral follicles of pre-pubertal animals have not been exposed to appropriate levels of gonadotropins, their follicles are not yet full size, and hence it becomes difficult to determine if donor age and follicle size have different effects on developmental outcome. Comparison of oocytes from different donor age groups and follicle sizes provides excellent models for studying oocyte maturation.

Cyclic adenosine monophosphate (cAMP) is an important second messenger involved in many pathways, and in particular is the principal regulator of the oocyte meiotic cell cycle. High levels of intra-oocyte cAMP maintain meiotic arrest by activating the protein kinase A (PKA) cascade of protein phosphorylation (Bornslaeger *et al.* 1986; Spaulding 1993; Francis and Corbin 1994). Increased intra-oocyte cAMP levels during

IVM have been reported to have uniform effects on the stage of meiotic maturation and to increase oocyte developmental potential (Mattioli *et al.* 1994; Funahashi *et al.* 1997b; Shimada and Terada 2002a; Somfai *et al.* 2003). Intra-oocyte cAMP levels throughout IVM may be important for successful oocyte maturation and subsequent development.

In this chapter, literature in the area of *in vitro* oocyte maturation is reviewed with particular reference to cAMP content, donor age and follicle size in the pig. The review first introduces follicle growth and development, and then discusses the endocrine and paracrine control of oocyte maturation *in vivo* and the regulation of the oocyte meiotic cell cycle *in vitro*. Finally, the effects of maternal donor age and follicle size on oocyte maturation are reviewed.

1.2 The ovarian follicle

1.2.1 Follicle growth and development in the pig

In the pig embryo, the ovary is first visible at 24-26 days after mating, with the primordial germ cells that make up the ovary visible by day 18 post-conception (Black and Erickson 1968). Approximately 500, 000 primordial follicles are present in each pig ovary 10 days postpartum (Black and Erickson 1968; Cardenas and Pope 2001). Germ cell mitotic division occurs from day 13 of embryonic life and continues up until 7 days after birth. Meiosis begins as early as day 40 of embryonic life and by around 35 days after birth all oogonia are in the prophase of the first meiotic division – germinal vesicle (GV). Oocyte growth progresses during folliculogenesis until the species dependent size has been reached, pig oocytes grow from 20 μM to 120 and 160 μM (zona-free and zona-intact respectively) (Morbeck *et al.* 1992; Bagg *et al.* 2004). From the primordial to primary follicle stage the granulosa cell appearance changes from flat to cuboidal

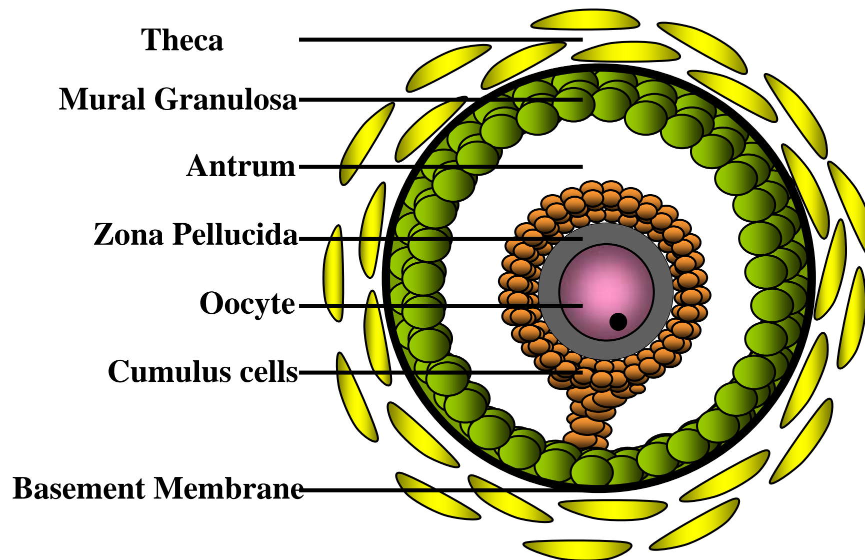
(reviewed by Picton 2001). During pre-antral (secondary) follicle development the oocyte continues to grow and acquire more granulosa cell layers (reviewed by Wassarman 1988). In the antral (tertiary) follicle, the granulosa cell layers separate with the formation of a fluid filled antrum (reviewed by Zamboni 1974 and Wassarman 1988). The first antral follicle has been observed at 70 days after birth in the pig, with development from the primordial to antral follicle stage taking around 84 days and follicle size increasing from 0.8 to 1.6 mm (Motlik *et al.* 1984; Morbeck *et al.* 1992; Hunter 2000). The antral follicle continues to grow due to increasing somatic cell proliferation and antrum size (Clark *et al.* 1975; Grant *et al.* 1989; Cardenas and Pope 2001). The pig oocyte attains meiotic competence 14 days post antrum formation in follicles >3 mm and with continued growth the follicle becomes pre-ovulatory (10 mm) in a further 19 days (Motlik *et al.* 1984; Morbeck *et al.* 1992; Hunter 2000).

Puberty, the time when mating and conception can first occur naturally in the pig, is coincident with the first oestrous and ovulation. In Australia, pigs generally achieve puberty by 27 weeks of age (5-6 months), allowing for puberty stimulation around 23 weeks of age (Hughes 2007). The oestrous cycle is responsible for: Follicle development from pre-ovulatory stage to ovulation and release of mature oocytes, blockage of further ovulation and preparation of the uterus to accommodate embryos as a result of fertilisation, or in the absence of fertilisation, luteolysis, the development of other follicles for a new oestrous cycle and ovulation. The pig oestrous cycle lasts 21 days and is comprised of a luteal phase and a follicular phase, which last around 16 and 5 days respectively. Follicle growth beyond the pre-ovulatory stage, oestrous and ovulation are dependent on the gonadotropins, follicle stimulating hormone (FSH) and LH, the mechanisms of which will be discussed in section 1.2.4.

1.2.2 Antral follicle components

In vivo, oocyte maturation takes place within the follicle as a result of major changes to its cellular microenvironment, changes regulated by a variety of hormones, but in particular the gonadotropins. Somatic follicle cells have been shown to have a positive effect on oocyte maturation, fertilisation and/or embryo development in a wide range of species (Armstrong *et al.* 1991). As figure 1.1 depicts, the antral follicle contains two anatomically and functionally different types of somatic granulosa cells; the mural granulosa cells that line the inner layer of the follicle wall; and the cumulus cells which enclose the oocyte (reviewed by Wassarman 1988). Since the cumulus cells are in closest contact with the oocyte, referred to together as the cumulus-oocyte complex, the oocyte is most sensitive to changes in the cumulus cells (Buccione *et al.* 1990; Driancourt and Thuel 1998). The corona radiata comprises the innermost cumulus cell layer and has the most extensive metabolic contact with the oocyte. Factors secreted by the granulosa cells are deposited in the follicular fluid (FF) surrounding the cumulus-oocyte complex (COC), and mediate oocyte growth and survival, and as such play an important role in follicle growth (reviewed by Picton 2001). In addition to products secreted by the follicle cells, FF also contains proteins and other factors derived from blood plasma (Edwards 1974; McNatty and Baird 1978). Maturation activating factors present in the FF are believed to exert a positive effect on oocyte cytoplasmic maturation, and hence FF is a common component in porcine IVM and will be discussed further in this review.

Figure 1.1 Schematic representation of the antral follicle components. Original artwork adapted from text descriptions by Wassarman, 1988.



1.2.3 Follicle cell communication

Ovarian folliculogenesis and oocyte maturation rely upon complex regulatory mechanisms involving endocrine and paracrine signalling pathways between the granulosa cells and the oocyte. Heterologous gap junction communication (GJC) increases during folliculogenesis from as early as the primordial follicle stage and facilitate communication between the cumulus cell layers and the oocyte (Albertini and Anderson 1974; Anderson and Albertini 1976; Gilula *et al.* 1978). The corona radiata layer of cumulus cells extends cytoplasmic processes through the zona-pellucida to form gap junctions with the oocyte surface (Albertini and Anderson 1974; Anderson and Albertini 1976; Gilula *et al.* 1978). Follicle cells are able to communicate directly with the oocyte and also with other cells via GJC (reviewed by Albertini *et al.* 2001). These interactions enable the follicular cells to supply the maturing oocyte with nucleotides, amino acids and phospholipids, while maintaining ionic balance and mRNA stability (Gilula et al. 1978; Heller and Schultz 1980).

The functional gap junction channel comprises two end-to-end intercellular membrane channels from two adjacent cells which enable passage of small (<1kDa) ions, metabolites and secondary messengers between cells through passive diffusion (Bruzzone *et al.* 1996a; Bruzzone *et al.* 1996b; Kidder and Mhawi 2002). These intercellular membrane channels, called connexons, are hemichannels in the cellular plasma membrane (reviewed by Kidder and Mhawi 2002). A connexon represents a hexamer of connexin proteins, each connexin belonging to a large group of integral membrane proteins (reviewed by Kidder and Mhawi 2002). Connexins are the product of distinct gene sequences and their identification number is defined by their molecular weight, with multiple connexins able to be expressed in the ovarian follicle and even in the same cell types within the follicle (reviewed by Kidder and Mhawi 2002). Five members of the connexin (Cx) gene family have been reported in pig ovarian follicles: Cx 26, Cx 30.3, Cx 32, Cx 43, and Cx 60 (Itahana *et al.* 1996; Itahana *et al.* 1998). Cxs 43 and 37 are the most abundant Cxs in the ovary, with Cx 43 found both on the surface of granulosa cells and the oocyte (Risek *et al.* 1990; Granot *et al.* 2002) and Cx 37 on the oocyte (Simon *et al.* 1997). The gap junctions that join cumulus cells are most commonly formed from Cx 43 (Beyer *et al.* 1989). Cx 43 expression increases in granulosa and cumulus cells in response to FSH, reaching the highest levels of expression at the antral follicle stage (Wiesen and Midgley 1993; Granot and Dekel 1997; Sommersberg *et al.* 2000). Cx 37 is also required by the oocyte for growth and maturation, and Cx 37 deficient mice are infertile (Simon *et al.* 1997) as a result of their inability to initiate meiotic maturation (Carabatsos *et al.* 2000).

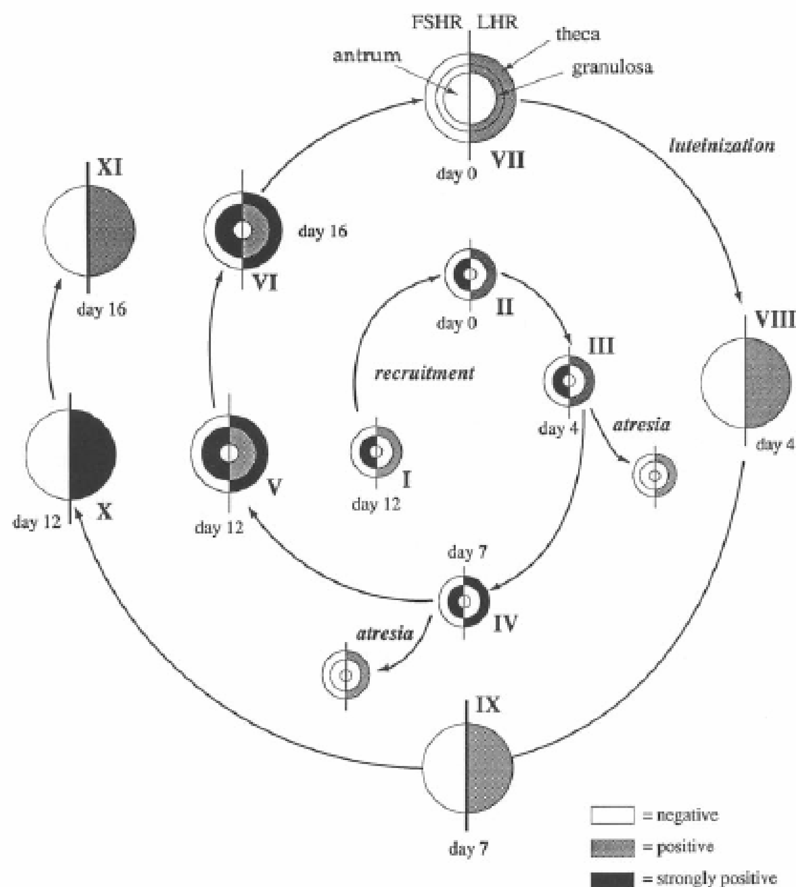
1.2.4 Gonadotrophic control

It has been well established that follicle growth beyond the secondary stage, the oestrous cycle, oocyte maturation and ovulation is dependent on the gonadotropins,

FSH and LH (Foxcroft and Hunter 1985; Esbenshade *et al.* 1990; Greenwald and Roy 1994). FSH and LH bind to their receptors on granulosa cells to stimulate adenylate cyclase production of cAMP, which plays an intermediary role in the induction of gene transcription for maturation via cAMP dependent protein kinases, such as protein kinase A (PKA) (Armstrong *et al.* 1991; Richards 1994; Richards *et al.* 1998). The gonadotropin responsiveness of a developing follicle depends on the number of specific receptors in various follicle cell types (Liu *et al.* 1998). Figure 1.2 illustrates cycle and maturation-dependent changes in gonadotropin receptors in the pig ovary. Since pigs are multiple ovulators, development of a number of follicles is synchronized throughout the oestrous cycle by heterogenous gonadotropin receptor expression (Liu *et al.* 1998). FSH is essential for the growth of small follicles (< 2mm) and increasing levels of LH are necessary for follicle growth beyond 2 mm in the pig (Driancourt MA 1995). FSH priming is required to increase LH receptor (LHR) mRNA expression and upregulate LHR formation in the ovarian follicle (Black and Erickson 1968; Zeleznik *et al.* 1974; Segaloff *et al.* 1990; Piquette *et al.* 1991; Shi and Segaloff 1995; Shimada *et al.* 2003b).

The timing and cell specific localization of the FSH receptor (FSHR) fits with its function in both early follicle development and in the induction of LHR expression as the follicle approaches ovulation. FSHR localize exclusively to the granulosa cell population within the follicle (Charlton *et al.* 1982; Oxberry and Greenwald 1982; Shima *et al.* 1987). FSH binding is follicle size dependent, relating to the quantity of receptors expressed in the granulosa cell population (Uilenbroek and Richards 1979). In the pig, expression of FSHR mRNA and FSH binding is greater in small follicles and adenylate cyclase responsiveness to FSH decreases with follicle growth (Lee 1978; Nakano *et al.* 1983; LaBarbera 1994; Yuan *et al.* 1996).

Figure 1.2 Cycle and follicle size dependent changes to FSHR and LHR in the pig ovary, complete diagram taken from Liu et al., (1998). The circles represent follicles, with the inner circles representing follicle cell types: outer - theca, intermediate – mural granulosa cell, inner - antrum & COC; with solid circles representing corpora lutea. FSHR is represented on the left and LHR on the right, with the stages of follicle maturation numbered I-XI in a 21-day oestrous cycle in which day 0 is oestrus.



The distribution of LHR in the ovary differs substantially compared with FSHR, the timing and localization of LHR supports its role in stimulating mural granulosa cell luteinisation, oocyte maturation and ovulation. LHR localize to thecal cells in the immature, pre-antral follicle and to both thecal and granulosa cells in mature, pre-ovulatory follicles (Zeleznik *et al.* 1974). In the pig, LHR are also present on the granulosa cells of small follicles (< 3 mm), but at a twentieth of the levels observed in

larger, pre-ovulatory follicles (Channing and Kammerman 1973; Lee 1976). LHR binding and adenylate cyclase responsiveness to LH also increases with increasing follicle size (Lee 1978; Yuan *et al.* 1996). In IVM pig oocytes, Shimada et al., 2003 elegantly demonstrated that treatment with FSH could increase LHR mRNA on the cumulus cells and binding of human chorionic gonadotropin (hCG) to COCs, which while holding oocytes in meiotic arrest proved to be a beneficial way to produce IVM oocytes with high developmental competence (Shimada *et al.* 2003b). LHR occupy the mural granulosa cell layer of the follicle and are absent or minimal on the cumulus cell layer and oocyte (Peng *et al.* 1991; Eppig *et al.* 1997), meaning LH mediates meiotic resumption in an indirect fashion. LH signalling appears to either remove a meiotic arresting factor(s) or supply maturation promoting factor(s) to the oocyte, but how this signal is transmitted to the oocyte from the mural granulosa cell layer is not well understood. Epidermal growth factor (EGF)-like proteins appear to induce maturation of follicle-enclosed oocytes and COCs, but have no effect on denuded oocytes (DOs) (Park *et al.* 2004; Ashkenazi *et al.* 2005). The LH mediated signalling pathway between cumulus cells and the oocyte is still not entirely understood in mammalian species. In amphibian oocytes, gonadotropins stimulate synthesis of steroid hormones, particularly progesterone and androgens, which then trigger oocyte maturation (Masui and Clarke 1979; Schmitt and Nebreda 2002; Thomas *et al.* 2002a; Tsafriri *et al.* 2005)

1.2.5 Steroids in oocyte maturation

Treatment of pig COCs with FSH and LH has been shown to induce progesterone receptor (PR) expression in cumulus cells along with increased progesterone production (Shimada and Terada 2002b). The addition of progesterone to the IVM media or stimulation to increase progesterone production by the COC, stimulates meiotic

resumption of porcine and bovine oocytes in both the presence or absence of gonadotropins (Racowsky 1985; Sirotkin 1992; Eroglu 1993; Shimada and Terada 2002b; Yamashita *et al.* 2003). Recently, FSH followed by LH stimulation of porcine COCs was reported to stimulate high levels of progesterone and cAMP production in cumulus cells followed by a loss in proliferative activity of cumulus cells and accelerated GV breakdown (GVBD), suggesting progesterone induced cumulus cell changes are involved in the induction of meiotic resumption (Okazaki *et al.* 2003).Yet, other studies in the pig, guinea pig and mouse report that progesterone has no effect or even an inhibitory effect on meiotic resumption (McGaughey 1977; Dekel and Kraicer 1978; Racowsky and McGaughey 1982b; Schultz *et al.* 1983b; Kaji *et al.* 1987; Racowsky and Baldwin 1989b; Barrett and Powers 1993; Yding Andersen and Byskov 2002). *In vitro*, FSH and LH triggered GVBD by increasing progesterone and progesterone receptor (PR) synthesis in porcine COCs (Shimada and Terada 2002b). A recent study in the pig reported that FSH and LH cause a PR isoform change from PR-B to PR-A, which appears to mediate the decrease in cumulus cell Cx 43 expression and cAMP level that is associated with resumption of oocyte maturation (Shimada and Terada 2002b). High levels of PI 3-kinase in cumulus cells also appears to be required for induction of gonadotropin-stimulated meiotic resumption (Shimada *et al.* 2003a). A possible candidate for stimulating meiosis appears to be follicular-fluid meiosis activating sterol (4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol; FF-MAS) (Schroepfer 1982), an intermediate in the cholesterol biosynthetic pathway, which increases following hCG stimulation and stimulates meiotic resumption of mammalian oocytes (reviewed Byskov *et al.* 2002 and Tsafriri *et al.* 2005). However, since FF-MAS is expressed after GVBD, it does not appear to be the initiating factor in meiotic resumption (reviewed Byskov *et al.* 2002 and Tsafriri *et al.* 2005).

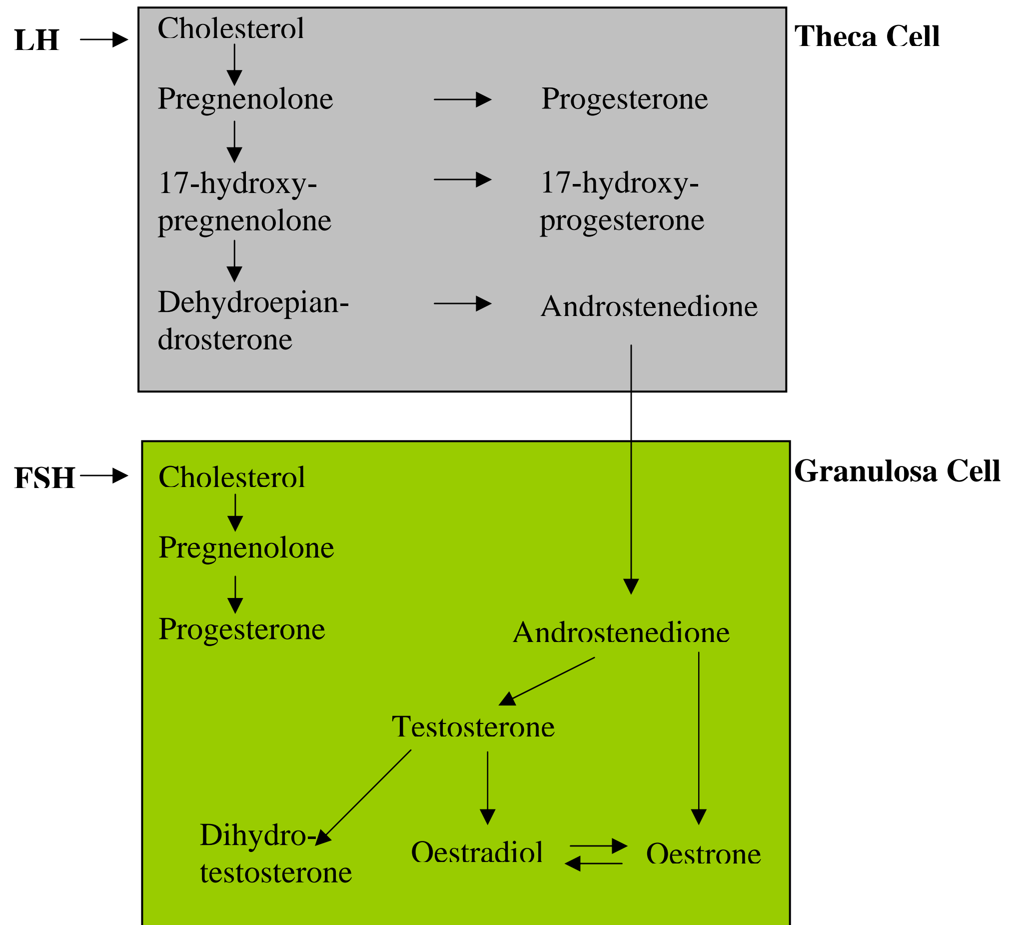
Growing follicles secrete 17β-oestradiol, which in turn stimulates the secretion of LH which is required for continued follicle growth to the pre-ovulatory size. Upon reaching this size, follicles are unable to ovulate and become atretic if progesterone is present, However, if there is no progesterone the large amount of 17β-oestradiol present will induce a LH surge that will cause ovulation approximately 42 h later. Follicle development occurs throughout the oestrous cycle, but the effects of 17β-oestradiol are only evident during the last 4-5 days of the follicular phase when the progesterone levels are low. The luteal phase is characterized by high rates of progesterone and 17β-oestradiol secretion from the CL. By day 16 in the absence of pregnancy, the uterus secretes prostaglandin PGF2 alpha to induce luteolysis and a drop in progesterone secretion (reviewed by (Przala *et al.* 2006).

The changing levels of FSH and LH provide important cues for induction of steroid production by theca and granulosa cells in the follicle in what is referred to as the two-cell, two gonadotropin model (see figure 1.3). Under the influence of LH, cholesterol is converted to pregnenolone and metabolised to yield androgen (Fortune 1986; Drummond 2006). Androgen in turn is transported from the theca to the granulosa cells where it can be aromatized to 17β-oestradiol (reviewed by Fortune 1986, Gore-Langton and Armstrong 1988, and Drummond 2006). Steroids signal through ligand-specific nuclear receptors to regulate transcriptional events and participate in feedback regulation of gonadotropin secretion by the pituitary and regulate pre-ovulatory development and function in autocrine and paracrine fashions (Fortune 1986). Of particular relevance to porcine follicle development are progesterone and 17β-oestradiol which accumulate in the FF at high concentrations and change markedly during follicle development (Eiler and Nalbandov 1977; Ainsworth *et al.* 1980).

Expression of the PR in granulosa cells increases with follicle size and can be induced by either FSH or LH via a cAMP mediated pathway (Natraj and Richards 1993; Park-Sarge and Mayo 1994). PR expression is highest in large antral follicles and absent in granulosa cells from small antral follicles in the pig (Slomczynska *et al.* 2000). Increased progesterone and PR expression in the cumulus cell layer is associated with meiotic resumption in the pig (Yamashita et al 2003, Shimada and Terada 2002). Rodent studies demonstrate that inhibition of progesterone synthesis blocks LH-induced ovulation *in vivo* and maturation *in vitro* and PR knockout mice are infertile (Loutradis *et al.* 1991; Uilenbroek *et al.* 1992; Lydon *et al.* 1995). Likewise, progesterone agonists inhibit meiotic resumption and cumulus expansion of pig COCs during IVM (Shimada 2004). *In vitro*, progesterone also acts on granulosa cells to enhance progesterone production, inhibit oestrogen production, slow the rate of mitogen induced proliferation and inhibit apoptosis (reviewed by Drummond 2006). These studies demonstrate that progesterone plays an important role in follicular development, oocyte maturation and ovulation.

Follicular 17β-oestradiol is highest in pre-ovulatory follicles and signals through oestrogen receptors (ER) in the granulosa cells (reviewed by (Drummond 2006). 17β-Oestradiol facilitates granulosa cell differentiation, induces expression of receptors for LH, FSH and prolactin, and regulates post-receptor mechanisms. Therefore, in the presence of FSH and LH, 17β-oestradiol stimulates cAMP accumulation in the granulosa cells (Richards *et al.* 1979; Richards and Rolfes 1980). ER knockout mice are not infertile but display severely reduced follicular development and 17β-oestradiol depleted ovaries undergo sex reversal (reviewed by Drummond 2006). These studies highlight the role of 17β-oestradiol in pre-ovulatory follicle formation from the antral stage onwards (reviewed by Drummond 2006).

Figure 1.3 Schematic of steroid biosynthesis in the ovary, adapted from relevant figures and literature (Fortune 1986, Gore-Langton and Armstrong 1988, and Drummond 2006).



1.2.6 Paracrine control

The growth/differentiation factors produced by the theca, granulosa cells and oocyte itself that have been implicated in folliculogenesis so far include the insulin-like growth factors (IGF-I & II), IGF binding proteins (IGF-BPs), epidermal growth factor (EGF), transforming growth factor (TGF) α, kit ligand/stem cell factor (KL/SCF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), tumour necrosis factor α (TNFα), follistatins, members of the (TGF) β superfamily such as inhibin, activin,

growth differentiation factor-9 (GDF-9) and bone-morphogenic protein (BMP15/GDF9b), as well as numerous other cell specific growth factors, cytokines and chemokines (Hillier 2001; Nilsson and Skinner 2001; Hunter *et al.* 2004; Conti *et al.* 2006; Knight and Glister 2006).

The IGFs and IGF-BPs regulate steroid synthesis in a paracrine manner, with the different follicle cells expressing genes for IGF-I, IGF-II and IGF-BPs in a developmentally regulated manner (Hillier 2001). IGF expression in response to gonadotropin stimulation appears to have a physiological paracrine role in regulating androgen production, while the IGF-BPs appear to locally mediate/modulate gonadotropin action (Hillier 2001). Numerous studies have implicated EGF in growth and differentiation of the ovarian follicle and steroidogenesis and in particular suggest LH stimulation of the ovarian follicle required activation of the EGF network. The EGF network includes EGF-like proteins that appear to play similar roles to EGF, to date these include TGFα, Heparin-binding EGF-like growth factor (HB-GF), amphiregulin (AREG), epiregulin (EREG), betacellulin (BTC), epigen, and neuroregulins (Conti *et al.* 2006). Regulation of granulosa cell growth in particular appears to be regulated by TGFα, TGFβ, keratinocyte growth factor and hepatocyte growth factor. Kit ligand/stem cell factor (KL/SCF), produced by immature granulosa cells, and FGF, produced mainly by the oocyte, seem to induce primordial follicle development and to influence puberty and menopause via their impact on theca cell organisation (Nilsson and Skinner 2001). Regulation of follicular angiogenesis appears important for ovulatory follicle development, particularly via VEGF, which is predominantly produced by granulosa cells and is gonadotropin responsive (Hunter *et al.* 2004). *In vitro* studies on isolated follicles, granulosa cells, theca cells and oocytes suggest that activins, follistatin and to a lesser degree, inhibin synthesised by granulosa cells exert local autocrine-paracrine

actions to modulate follicle growth, gonadotropin responsiveness, steroidogenesis, oocyte maturation, ovulation and CL function (Knight and Glister 2006). Expression of TGFβ superfamily growth factors in ovarian somatic cells changes in a developmental, stage related fashion and function as intra-ovarian regulators of folliculogenesis processes such as follicle growth and transition to various follicle stages, granulosa cell proliferation, follicle survival, steroid production and gonadotropin action (Knight and Glister 2006).

1.3 Oocyte meiotic resumption

In order for fertilisation to occur, mammalian oocytes must first resume and complete meiosis. In the fetal ovary, meiosis progresses to the diplotene (dictyate) stage of prophase, with resumption occurring in response to hormonal stimulation by the gonadotropins (reviewed by Picton 2001). The majority of oocytes are then arrested at the MII stage of meiosis, which resumes following fertilisation or parthenogenetic activation. Meiotically arrested oocytes contain diffuse chromosomes surrounded by an intact nuclear structure known as the germinal vesicle (GV) which is characterised by an intact GV with a distinct nuclear envelope, a nucleolus surrounded with chromatin in the form of a ring or horseshoe and a finely granular nucleoplasm. The first meiotic resumption is characterised by GV breakdown (GVBD – GV stages I to IV), by the end of which the nuclear membrane is barely visible, the nucleolus has completely disappeared and the chromatin has formed an irregular network or individual filamentous bivalents. GVBD leads to diakinesis where the chromatin undergoes condensation into either single lumps or into smaller discrete fragments. At MI, chromatin bivalents are assembled at the spindle equator. As meiosis proceeds to anaphase (ana I), bivalents move to opposite ends of the spindle, aligning as homologous chromatid pairs, and are separated as the spindle moves 90°. Straight after

the separation, in telophase I (telo I), the oocyte and polar body chromosomes are similar in appearance, but the polar body chromosomes begin to degenerate. By MII, all polar body chromosomes have degenerated and are enclosed by polar body with the oocytes chromosomes aligned on a spindle. Descriptions of meiotic stages described here were taken from cited references (Motlik and Fulka 1976; Wassarman 1988).

1.3.1 Follicle cells & meiotic resumption

Given that the oocytes undergo spontaneous meiotic resumption upon liberation from the follicle (Pincus and Enzmann 1935) and that follicle-enclosed oocytes matured *in vitro* remain in meiotic arrest (Tsafriri and Channing 1975; Tsafriri 1979; Racowsky and McGaughey 1982a), follicle cells appear to play an important role in meiotic inhibition. Co-culture of COCs with granulosa or theca cell monolayers or with granulosa cell conditioned media has been shown to maintain meiotic arrest (Sirard and Bilodeau 1990a; Sirard and Bilodeau 1990b; Kotsuji *et al.* 1994; Richard and Sirard 1996b; van Tol and Bevers 1998). Hemi-sections and pieces of the follicle wall also have an inhibitory effect on meiosis, regardless of whether they are in contact with the oocyte (Racowsky and Baldwin 1989a; De Loos *et al.* 1994; Richard and Sirard 1996a). Hemisections from the granulosa cell layers were less potent at maintaining meiotic arrest of bovine oocytes in GV stage compared to the conditioned medium of two complete follicular hemisections (57% vs. 95%), in particular the thecal cells (interna or externa) appeared to maintain oocytes in the GV stage (Richard and Sirard 1996b; Richard and Sirard 1996a). A later bovine study demonstrated that inhibitory factor(s) produced by the thecal cells were soluble in media and act through the cumulus cells surrounding the oocyte to establish meiotic arrest (Richard and Sirard 1996b).

1.3.2 Follicular fluid & meiotic resumption

Follicular fluid has also been shown to maintain meiotic arrest to various extents in different species (Racowsky and Baldwin 1989a; Sirard and Bilodeau 1990b; Downs 1993). Pig and mouse FF was originally shown to maintain mouse oocytes in meiotic arrest, with the purines hypoxanthine and adenosine thought to be the FF components mediating this effect (Downs and Eppig 1986a). Purines have been shown to inhibit meiotic resumption of mouse (Downs *et al.* 1986b), rat (Tornell and Hillensjo 1993), cow (Sirard and First 1988) and pig (Miyano *et al.* 1995) oocytes. Purines are thought to mediate their inhibitory effect by inhibiting phosphodiesterase (PDE) enzymes (which degrade cAMP) to maintain intra-oocyte cAMP, but given the complexity of FF, other factors may also be involved (Aktas *et al.* 1995b; Downs 1997).

1.3.3 Gap Junction Communication & meiotic resumption

The loss of GJC between the cumulus cells and the oocyte has long been believed to facilitate meiotic resumption by interrupting the transfer of meiosis arresting signals from the follicle cells into the oocyte (Gilula *et al.* 1976; Dekel *et al.* 1981). There is some controversy as to whether GJC is disrupted prior to or during meiosis. Timing of GJC breakdown has been reported to coincide with meiotic resumption in the cow (Hyttel 1987), rat (Dekel *et al.* 1981; Sherizly *et al.* 1988) and pig (Isobe *et al.* 1998; Isobe and Terada 2001; Shimada *et al.* 2001). Conversly, GJC breakdown has been reported to occur after meiotic resumption in the mouse (Eppig and Downs 1984) and pig (Motlik *et al.* 1986). Supporting a link between GJC breakdown and meiotic resumption, cumulus expansion and GJC breakdown have also been reported to coincide with GVBD (Suzuki *et al.* 2000; Sato and Yokoo 2005). However, a number of studies have indicated that GJC is maintained between the cumulus cell layer and

oocyte even after GJC breakdown within the cumulus layer itself (Salustri and Siracusa 1983; Motlik *et al.* 1986; Eppig 1989; Buccione *et al.* 1990; Isobe *et al.* 1998). Consistent with this, persistence of GJC in the COC has been observed as late as 24 h IVM in the cow (Sutovsky *et al.* 1993) and until 36-44 h in the pig (Suzuki *et al.* 2000). These studies suggest that a disruption of cumulus-cumulus GJC but not cumulus-oocyte GJC facilitates meiotic resumption.

A recent study supports these disparate roles, observing a Cx43 reduction in the outermost layers of cumulus cells at the time of GVBD and within the innermost layers of cumulus cells surrounding the oocyte at the later time of metaphase I (MI) (Shimada *et al.* 2001). Reduction of Cx 43 mRNA and protein in the cumulus cell layer appears to be triggered by the same gonadotropins that induce meiotic resumption (Larsen *et al.* 1987; Wiesen and Midgley 1993; Granot and Dekel 1994; Granot and Dekel 1997). It appears that gonadotropins reduce Cx 43 levels by inhibiting translation of the protein via a phosphorylative pathway shown to involve PKA (Granot and Dekel 1994; Kalma *et al.* 2004), PKC (Granot and Dekel 1994; Shimada *et al.* 2001), phosphoinositide 3-kinase (PI-3 Kinase) (Shimada *et al.* 2001) and mitogen-activated protein kinase (MAPK) (Shimada and Terada 2001; Kalma *et al.* 2004). A recent study has elegantly demonstrated, using progesterone inhibitor RU486, that gonadotropins also induce a shift in PR isoforms along with an increase in progesterone that results in an increase in cumulus cell proliferation, a decrease in Cx 43 and meiotic resumption (Shimada *et al.* 2004).

There is also evidence that cAMP levels can regulate GJC between various cell types, in a number of tissues and species (Eppig and Ward-Bailey 1982; Furger *et al.* 1996; Ghosh and Singh 1997; Murray *et al.* 1998; Cruciani and Mikalsen 2002; Pant *et al.* 2005). The membrane permeable cAMP analogue, dbcAMP, has been reported to

increase intercellular GJC between somatic cell types in sheep and mouse studies (Eppig and Ward-Bailey 1982; Grazul-Bilska *et al.* 2001). Dibutyryl cAMP has also been reported to modulate number, size and distribution of gap junctions in adrenal and rat prostate tumour cells (Murray *et al.* 1998).

1.3.4 cAMP

It is well established that intra-oocyte cAMP concentrations regulate the meiotic cell cycle (Conti *et al.* 2002). The cAMP content in cells results from cAMP production by adenylate cyclase and cAMP degradation by phosphodiesterases (PDE). Intracellular cAMP was first implicated as an important regulator of cell cycle progression in early cell line studies, which showed a marked decrease in cAMP upon cell entry into mitosis from G_2, and fluctuation in cAMP levels amongst other mitotic phases (Masui and Clarke 1979). Treatment with cAMP analogues and PDE inhibitors also prevents interphase cells from entering mitosis (Masui and Clarke 1979). Many similarities have been drawn between the regulation of mitotic and meiotic cell cycles. Elevation of cAMP levels in frog and starfish oocytes using PDE inhibitors, cAMP analogues and activators of adenylate cyclase prevented progesterone-induced oocyte maturation, demonstrating cAMP to be an important regulator of meiosis (Masui and Clarke 1979). Furthermore, a decline in cAMP levels was consistently observed in mammalian oocytes isolated from follicles (Lindner *et al.* 1974; Dekel and Kraicer 1978; Dekel and Beers 1980; Schultz *et al.* 1983a; Eppig and Downs 1984).

1.3.4.1 Follicle sources of cAMP

Early studies employing forskolin to stimulate adenylate cyclase, the enzyme responsible for cAMP production, resulted in inhibition of meiotic resumption in rat COCs but not DOs *in vitro* (Dekel *et al.* 1984; Racowsky 1984). Treatment with

forskolin during maturation was also employed to demonstrate that the intact COC has a higher intra-oocyte cAMP content compared to oocytes denuded of their cumulus cells in the mouse, rat, pig and cow (Racowsky 1984; Racowsky 1985; Bornslaeger and Schultz 1985b; Bilodeau *et al.* 1993). Despite this, forskolin can stimulate cAMP production in DOs from the pig (Racowsky 1985), cow (Bilodeau *et al.* 1993), rat (Olsiewski and Beers 1983) and mouse (Bornslaeger and Schultz 1985a). Adenylate cyclase has only been cytochemically localised to the oolemma of the oocyte in the cow, and interestingly the presence of adenylate cyclase within cumulus cell projections increased when stimulated with forskolin (Kuyt *et al.* 1988). These results suggest that even though oocytes do appear to possess some enzymatic adenylate cyclase activity, this probably does not have a major impact on their maturation. Thus, the majority of studies suggest the follicular cells are responsible for synthesising the major portion of intra-oocyte cAMP.

A number of studies in a wide range of species have supported GJC as being the main passage for cAMP entry into the oocyte from the cumulus cells (Sela-Abramovich *et al.* 2006). Indeed, the permeability of gap junctions from a wide variety of tissues to cAMP has been well documented (Tsien and Weingart 1976; Pitts and Simms 1977; Lawrence *et al.* 1978; Fletcher and Greenan 1985).

1.3.4.2 Intra-oocyte cAMP measurements

A decrease in intra-oocyte cAMP levels precedes meiotic resumption in rat (Schultz *et al.* 1983a; Aberdam *et al.* 1987; Eppig 1989) and mouse oocytes (Vivarelli *et al.* 1983; Schultz *et al.* 1983a; Aberdam *et al.* 1987). Pig and rabbit oocytes differ from these and exhibit a transient increase in intra-oocyte cAMP following gonadotropin stimulation *in vivo* (Yoshimura *et al.* 1992b; Mattioli *et al.* 1994). However, there is some disagreement as to what happens to the cAMP level during IVM of pig oocytes. The

intra-oocyte cAMP did not change during IVM of follicle enclosed oocytes matured without LH or in COCs and DOs matured in the presence of LH (Mattioli *et al.* 1994). Mattioli et al. (1994) demonstrated that intra-oocyte cAMP levels of follicle-enclosed oocytes increased following administration of hCG *in vivo* or in the presence of LH *in vitro*, returning to basal (0h) levels by 24 h. Shimada and Terada (2002a) reported an increase in intra-oocyte cAMP in COCs from the start of IVM, peaking at 8 h and then steadily decreasing until 32 h. This and another study also reported that the cAMP concentration of intact COCs increased in the first half of IVM and remained high through to 48 h IVM (Racowsky 1985; Shimada and Terada 2002a). Thus, there is still uncertainty as to the concentration of intra-oocyte cAMP, and pattern of changes required during IVM for successful oocyte maturation and development.

1.3.4.3 Treatments to increase cAMP

Numerous studies, in a variety of mammalian species, have implicated high intra-oocyte cAMP in the maintenance of meiotic arrest. Spontaneous meiotic resumption of follicle liberated COCs can be prevented by a number of treatments that increase cAMP. Dibutyryl cAMP, a membrane permeable cAMP analogue, mimics the action of endogenous cAMP (Budavari 1989). In comparison with cAMP, dbcAMP has a lipophilic nature and is therefore used preferentially with intact cells due to its greater permeability and resistance to hydrolysis by PDEs (Henion et al. 1967; Swislocki 1970). Membrane permeable cAMP analogues such as dbcAMP and 8-Br-cAMP have been shown to maintain oocyte meiotic arrest in the mouse (Cho *et al.* 1974; Downs *et al.* 1989), rat (Magnusson and Hillensjo 1977), cow (Homa 1988; Sirard and First 1988; Aktas *et al.* 1995b), human (Tornell and Hillensjo 1993) and pig (Rice and McGaughey 1981; Mattioli *et al.* 1994; Funahashi *et al.* 1997b). Activators of adenylate cyclase such as forskolin, cholera toxin, prostaglandin, 17β-oestradiol and sodium fluoride induce

cAMP production in cumulus cells and increase intra-oocyte-cAMP (Racowsky 1985; Bilodeau *et al.* 1993). Forskolin in particular has been used extensively to increase intra-oocyte cAMP and maintain meiotic arrest in a wide range of species (Dekel *et al.* 1984; Racowsky 1984; Homa 1988; Yoshimura *et al.* 1992b), including the pig (Racowsky 1985). Likewise invasive adenylate cyclase has also been shown to increase oocyte cAMP and reversibly inhibit maturation of COCs and DOs *in vitro* (Aktas *et al.* 1995a; Luciano *et al.* 2004). Non selective PDE inhibitors such as 3-isobutyl-1-methyxanthine (IBMX) also increase intra-oocyte cAMP and inhibit meiotic resumption in a dose dependent manner in the rat (Cho *et al.* 1974; Schultz *et al.* 1983a; Downs *et al.* 1989), cow (Homa 1988; Sirard and First 1988; Aktas *et al.* 1995b), and pig (Fan *et al.* 2002; Laforest *et al.* 2005). Use of specific PDE inhibitors in the cow, against PDE 3 which mainly populates the oocyte and PDE 4 which mainly populates the cumulus cells, has demonstrated that oocyte cAMP levels are mainly controlled by PDE mediated degradation, while cAMP in the cumulus cell layer is predominantly controlled by active adenylate cyclase (Thomas *et al.* 2002b).

1.3.4.4 cAMP paradox

Cyclic AMP appears to play opposing roles during meiotic resumption, depending on whether the oocyte is enclosed within the whole follicle or the COC alone. In follicle-enclosed oocytes, agents that increase cAMP actually trigger meiotic resumption (Tsafriri and Kraicer 1972; Lindner *et al.* 1974; Hillensjo *et al.* 1978). Specifically, GVBD can be induced in follicle-enclosed oocytes by injecting the cAMP analogue dbcAMP into the antrum (Tsafriri and Kraicer 1972) or by transiently exposing follicles to another analogue 8-bromo-cAMP (Hillensjo *et al.* 1978). Transient exposure of cultured follicle-enclosed oocytes to dbcAMP, IBMX or forskolin was also able to trigger GVBD (Dekel *et al.* 1981; Dekel and Sherizly 1983; Yoshimura *et al.* 1992a;

Yoshimura *et al.* 1992b). It appears that high levels of cAMP in the follicle results in oocyte maturation, while high intra-oocyte cAMP prevents maturation. Compartmentalisation and differential regulation of cAMP in the oocyte and the follicle cell layers via action of specific PDEs has been proposed to explain these opposing roles of cAMP (Tsafriri *et al.* 1996; Thomas *et al.* 2002b).

1.3.4.5 MPF/MAPK

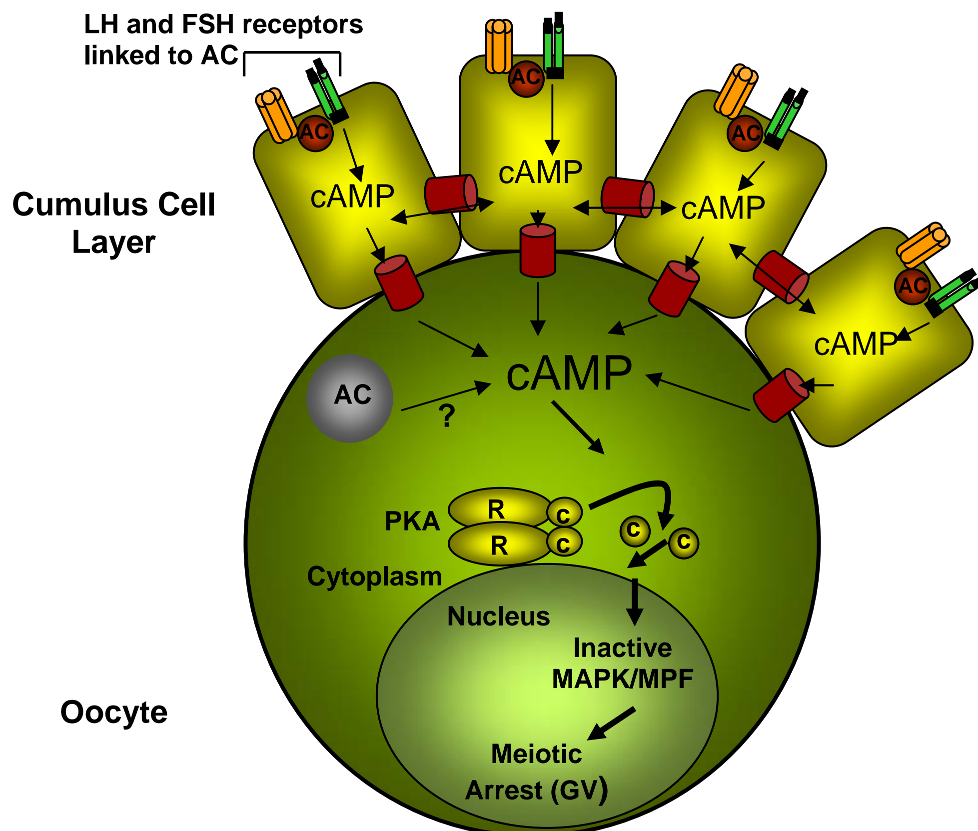
Ultimately, a cascade of specific phosphorylation/dephosphorylation biochemical events downstream of cAMP control meiosis in the mammalian oocyte by determining the activity of the maturation (or meiosis) promoting factor (MPF). At this time this cascade has not been completely elucidated, however a number of key players have been identified. Oocyte maturation results from a decrease in intracellular cAMP and subsequent inactivation of cAMP-dependent protein kinase A (PKA) triggering a cascade of phosphatases and kinases that ultimately activate MPF (Maller and Krebs 1977; Maller and Krebs 1980). High levels of cAMP during meiotic arrest maintain PKA in its active/dissociated state, in which the catalytic subunits are free to enter the nucleus of the oocyte and maintain MAPK and MPF inactive and thus maintain meiotic arrest (Bornslaeger *et al.* 1986; Spaulding 1993; Francis and Corbin 1994).

MPF is a heterodimer consisting of the catalytic p34cdc2 kinase and the regulatory cyclin B which becomes activated upon phosphorylation of p34cdc2 (Masui and Markert 1971; Nurse 1990). Oocytes in meiotic arrest (GV) display relatively low activity of MPF, which can be measured by examining levels of histone H1 enzyme activity (Dekel 1996). MPF activity then increases to maximum levels at MI and is followed by a sudden decrease in oocytes releasing the first polar body (Hashimoto and Kishimoto 1988; Choi *et al.* 1991; Dekel 1996; Zernicka-Goetz *et al.* 1997). Histone H1

activity increases again at MII and remains high until fertilisation or parthenogenetic activation (Dekel 1996).

Upstream of MPF, MAPK acts as intermediates in the signal-transduction pathway leading to MPF activation and are phosphorylated and activated during oocyte maturation in a number of species, including the pig (Gotoh *et al.* 1991; Sobajima *et al.* 1993; Inoue *et al.* 1995). Members of the MAPK family, ERK 1 and ERK 2, become active prior to MI and their activity remains high throughout oocyte maturation (Verlhac *et al.* 1993). Figure 1.4 demonstrates how high intra-oocyte cAMP is believed to inhibit meiotic resumption by maintaining MPF and MAPK in an inactive state (Choi *et al.* 1991; Lazar *et al.* 2002).

Figure 1.4 Schematic representation of signalling pathway for cAMP maintenance of meiotic arrest. Original artwork, adapted from text descriptions in recent reviews (Conti et al. 2002; Mehlmann 2005).



1.4 *In vitro* oocyte maturation

In vivo, maturation of oocytes in the pre-ovulatory follicle stage is triggered by the ovulatory LH surge. IVM media formulations aim to imitate the *in vivo* environment as closely as possible. IVF of oocytes matured *in vitro* was first achieved in 1974 for the pig and in 1980 for the cow (Motlik and Fulka 1974, Hunter *et al.* 1972). However, it was not until 1985 for the pig and 1989 for the cow that live offspring resulted from *in vitro* matured oocytes (Hanada *et al.* 1986; Mattioli *et al.* 1989). However, the developmental ability, fertilisation outcomes and embryo quality of oocytes matured *in vitro* were far inferior to those matured *in vivo*. Extensive research has led to successful attainment of MII (>90%) in current porcine and bovine IVM-IVF systems (Moor *et al.* 1990; Niwa 1993; Nagai 1994; Blondin *et al.* 1996). In the cow, despite similar rates of fertilisation and cleavage (Blondin *et al.* 1996), poor blastocyst formation rates have been reported for oocytes matured and fertilised *in vitro* (30-40 %) compared with those matured and inseminated *in vivo* (85%) (Greve *et al.* 1987; Leibfried-Rutledge *et al.* 1987; Sutton-McDowall *et al.* 2006). In the early days of pig IVM, extremely low blastocyst formation rates (for example, 1.4 %) were achieved (Nagashima *et al.* 1993). In these studies, the low developmental competence of IVM-IVF oocytes appeared to result from variability across ejaculates, a high incidence of polyspermy and low rates of male pronuclear formation (Motlik and Fulka 1974; Nagai *et al.* 1984; Mattioli *et al.* 1988a; Nagai and Moor 1990; Niwa 1993; Nagai *et al.* 1994). Extensive research into this area over the last decade has led to vastly improved fertilisation rates in the pig (Gore-Langton and T. 1988; Mattioli *et al.* 1989; Nagashima *et al.* 1993; Yoshida *et al.* 1993b; Funahashi *et al.* 1994; Nagai *et al.* 1994; Grupen *et al.* 1995; Nagashima *et al.* 1996; Prather and Day 1998; Abeydeera 2002). Poor blastocyst formation rates were reported for IVM-IVF oocytes compared with those matured and fertilised *in vivo* (11%

vs. 42%; (Nagashima *et al.* 1996). These findings suggest that IVM systems are deficient. Since nuclear maturation appears normal, it appears that the cytoplasmic maturation of *in vitro* matured oocytes may be incomplete.

Even stringent follicle and oocyte selection does not always yield oocytes capable of blastocyst development following IVM-IVF. Pig oocytes for IVM studies are generally aspirated from 3-8 mm antral follicles, in which the oocyte is considered to have achieved full growth and meiotic competence (reviewed by Nagai 2001). Morphologically, COCs with at least 3 layers of cumulus cells are generally selected for IVM (reviewed by Nagai 2001). An extensive list of nuclear and cytoplasmic factors have been shown to improve pig oocyte maturation *in vitro* when included as components in the IVM system and are presented in Table 1.1 (Hunter 2000). These components can either act directly on the oocyte or indirectly via the cumulus cell layer, which is present during pig IVM.

Table 1.1 Summary of media components shown to improve pig oocyte IVM (Modified and extended from review by Hunter, 2000).

NOTE: This table is included on page 45 of the print copy of the thesis held in the University of Adelaide Library.

1.4.1 Nuclear maturation

Nuclear maturation refers to the ability of the oocyte nucleus to progress from the GV stage to MII stage of meiosis. Meiotic ability in all species studied, including the pig, increases with increasing oocyte and follicle diameter, with oocytes < 100μm and follicles < 3 mm considered to be meiotically incompetent in the pig (Marchal *et al.* 2001; Lucas *et al.* 2002). When hCG was injected to induce the meiotic resumption of pig oocytes *in vivo*, GVBD was observed after about 18 h and meiosis was completed by 36 to 40 h post gonadotropin exposure (Hunter and Polge 1966). IVM culture closely follows these time periods in the pig, with a period between 36 and 48 h used to allow progression to MII. Gonadotropin stimulation and the presence of the cumulus layer for the first 20 h of IVM was shown to result in high rates of MII at the end of IVM (Funahashi and Day 1993; Kameyama and Ishijima 1994). High rates of nuclear maturation (>90 %), similar to those attained *in vivo*, are now achieved by the end of IVM in many different culture systems and media varieties (Moor *et al.* 1990; Niwa 1993; Nagai 1994). However, high nuclear maturation does not guarantee the oocyte is of good quality, and often only a small percentage (25-30% in the pig) of oocytes will develop to the blastocyst stage.

A large variation exists in the GV stage (between I and IV) of oocytes collected from a range of antral follicles both at the time of collection and after defined periods of IVM (Grupen *et al.* 1997; Nagai *et al.* 1997; Funahashi *et al.* 1997a). This variation appears to be a result of differences in GV status in oocytes obtained from different follicle sizes within the aspiration range (McGaughey *et al.* 1979; Grupen *et al.* 1997). Differences in the GV stages at 0h are likely due to the aspiration of some atretic antral follicles that are at various stages of GVBD and maturation *in vivo* prior to degeneration (Himelstein-Braw *et al.* 1976). Continued asynchronous meiotic progression throughout IVM gives

rise to a population of aged oocytes which have different developmental abilities following embryo IVP (McGaughey and Polge 1972; Motlik and Fulka 1976; Funahashi and Day 1993; Ocampo *et al.* 1993; Christmann *et al.* 1994; Grupen *et al.* 1997). Increases in intra-oocyte cAMP using dbcAMP during IVM was found to slow meiotic maturation and promote a more synchronous meiotic progression to MII (Funahashi *et al.* 1997b).

Mattioli et al. (1994) demonstrated that 1 mM dbcAMP could inhibit oocyte GVBD in a dose dependent manner and could even inhibit meiotic progression when added 10 and 20 h after the start of IVM (Mattioli *et al.* 1994). Treatment of porcine oocytes for the first 20 to 22 h of IVM with 1 mM dbcAMP has been shown to inhibit GVBD (Funahashi *et al.* 1997b; Somfai *et al.* 2003). Dibutyryl cAMP has been shown to improve the rate of blastocyst formation (Funahashi *et al.* 1997b; Somfai *et al.* 2003) and monospermic fertilisation (Somfai *et al.* 2003), but has no effect on sperm penetration and male pronuclear formation (Funahashi *et al.* 1997b; Somfai *et al.* 2003). Transfer of embryos derived from oocytes matured in the presence of dbcAMP has resulted in pregnancies and the birth of live piglets (Funahashi *et al.* 1997b). These findings demonstrate that co-ordination of nuclear maturation by increasing intra-oocyte cAMP artificially is an effective way to improve meiotic and developmental competence of pig oocytes.

1.4.2 Cytoplasmic maturation

Although not as well defined as nuclear maturation, cytoplasmic maturation encompasses many changes that are required to prepare the oocyte for activation, fertilisation and development. These changes occur at the level of mRNA transcription (Hunter and Moor 1987; Kastrop *et al.* 1991), protein translation (Sirard *et al.* 1989), post-translational modification of proteins (Levesque and Sirard 1994) and as

ultrastructural modifications (Greve *et al.* 1987). Mechanisms that have already been implicated in cytoplasmic maturation include sequestration of calcium for oocyte activation, cortical granule migration and preparation of machinery involved in pronuclear formation and cleavage to the blastocyst embryo stage (reviewed by Armstrong 2001).

Release of intracellular calcium is important for activation of the oocyte and the release of cortical granules to block against polyspermy (Ducibella *et al.* 1988; Fujiwara *et al.* 1993; Eppig 1996). The amount of calcium and the number of smooth-membrane vesicles that store the calcium increase dramatically in mature oocytes compared with those in GV stage (Ducibella *et al.* 1990; Eppig 1996). Likewise, exocytosis of cortical granules in response to calcium ionophore increases with the progression of nuclear maturation (Ducibella *et al.* 1990; Eppig 1996). These changes in calcium, as well as number and location of cortical granules within the oocyte facilitate monospermic fertilisation and sperm processing at the time of fertilisation.

Glutathione (GSH) also appears to be involved in sperm decondensation as well as oocyte activation and transformation of the penetrated sperm head into the male pronucleus (MPN) (Perreault *et al.* 1988; Yoshida *et al.* 1993a; Eppig 1996; Tanghe *et al.* 2002). Concentrations of GSH increase during oocyte maturation and when this process is inhibited then MPN formation does not occur (Perreault *et al.* 1988; Yoshida *et al.* 1993a). GSH also appears to protect cells against oxidative stress by maintaining its redox state (Grupen *et al.* 1995; Nagai 2001; Tanghe *et al.* 2002). In the pig in particular, the cumulus cells enhance the GSH content of the oocyte, thereby playing a critical role in protecting against oxidative injury (Tatemoto *et al.*, 2000; Tanghe *et al.* 2002). Addition of GSH precursors, cysteamine and cysteine, have been shown to

increase MPN and subsequent embryo development of pig and bovine oocytes *in vitro* (Grupen *et al.* 1995; Eppig 1996; de Matos *et al.* 1997; Maedomari *et al.* 2007). In pig DOs this approach did not improve their developmental competence, thus demonstrating that cumulus cells also mediate the positive effect of cysteamine addition (Yamauchi and Nagai 1999). Protein synthesis also appears important for successful fertilisation of the oocyte, since decondensation of the sperm nucleus to form the male pronucleus is severely impaired when protein synthesis is inhibited during oocyte maturation (Ding *et al.* 1992; Eppig 1996). It is likely that specific proteins are required for the reduction of the disulfide bonds in protamine, which is necessary for the oocyte to extract and/or degrade the protamines found in the sperm nucleus and replace them with histones (see Eppig 1996 for review). Thus, synthesis of specific proteins also appears a requirement of MPN production following sperm penetration.

The cumulus cells play an important role in cytoplasmic maturation, with increased numbers of cumulus cell layers and COC compactness pre-IVM correlated with improved developmental outcome in the cow (Shioya *et al.* 1988) and the pig (reviewed by Abeydeera, 2002). The presence of the cumulus cells during IVM is essential to subsequent developmental competence in both species (Shioya *et al.* 1988; Hashimoto *et al.* 1998). In the pig, the presence of cumulus cells during IVM has a positive effect on meiotic maturation, intracellular GSH concentration, sperm penetration, pronuclear formation, and histone H1 kinase activity (Yamauchi and Nagai 1999). Cumulus cells also appear to impact on the correct distribution and migration of cortical granules and thus the block to polyspermy (Yamauchi and Nagai 1999). The degree of cumulus expansion as IVM progresses is also thought to indicate successful maturation in most species, since the mature oocyte is surrounded in an expanded, mucified mass of cumulus cells at the time of ovulation *in vivo* (Hashimoto *et al.* 1998; Yokoo and Sato

2004). After the gonadotropin surge, cumulus cells organise a muco-elastic extracellular matrix that requires a large amount of hyaluronin and hyaluronin-binding matrix glycoproteins, which appears to play an important role in both meiotic and cytoplasmic maturation of the oocyte (Kimura *et al.* 2006). Cumulus cells also appear to assist the oocyte via glucose uptake and conversion to forms such as pyruvate or krebs cycle intermediates that can enter and be used by the oocyte (Biggers *et al.* 1967; Eppig 1996). The cumulus cells may also exert a positive influence on oocyte maturation by lowering the oxygen tension in the immediate vicinity of the oocyte (Kikuchi *et al.* 2002; Tanghe *et al.* 2002).

Optimal supplementation of the IVM media is also important for complete cytoplasmic maturation of the oocyte. Successful IVM of pig oocytes has been achieved using both simple and complex medias which contain a protein source such as fetal calf serum or FF supplementation, plus other supplements including gonadotropins and growth factors (reviewed in Abeydeera 2002). A number of *in vitro* studies indicate that addition of EGF to IVM media can improve both nuclear and cytoplasmic maturation in a number of animals including the pig. In two studies, media supplementation with EGF did not alter meiotic maturation, yet increased the rate of male pronuclear (Ding and Foxcroft 1994) and blastocyst (Illera *et al.* 1998) formation in the pig, suggesting its importance to cytoplasmic maturation. Li et al. (2002) demonstrated that EGF acts synergistically with 17β-oestradiol to significantly enhance nuclear and cytoplasmic maturation by increasing the rate of oocyte maturation to MII, forming MPN, undergoing embryo development and increasing the intracellular glutathione synthesis (Li *et al.* 2002). Lower follicular concentrations of progesterone and 17β-oestradiol have been correlated with poor developmental outcomes in the pig (Liu *et al.* 2002). However, the relevance of using FF steroid levels to estimate oocyte cytoplasmic

maturity and subsequent blastocyst development is contentious as the effect of steroids on oocyte maturation is not completely defined and varies amongst species. Addition of progesterone to IVM has been shown to increase IVF outcomes (Mattioli *et al.* 1998) and cytoplasmic maturation (Karlach 1987; Zhang and Armstrong 1989) of pig oocytes; while other studies in the pig (Dode and Graves 2002) and also the cow (Sirotkin 1992) report that progesterone has no influence on these parameters.

1.5 Developmental competence

The developmental competence of an oocyte refers to its meiotic competence, ability to cleave following fertilisation, develop to the blastocyst stage, induce a pregnancy and yield healthy offspring (reviewed by Sirard *et al.* 2006). Developmental competence is acquired gradually during oocyte growth and up until the final stages of follicle development prior to ovulation (reviewed by Krisher *et al.* 1999). Oocytes matured *in vitro* have lower developmental competence than ovulated oocytes (reviewed by Krisher *et al.* 1999). *In vitro* conditions do not precisely duplicate the *in vivo* environment, thus it is likely that the current IVM procedure lacks factor(s) required for successful maturation compared with that *in vivo*.

Fertilised oocytes developing to the blastocyst embryo stage have a significant chance of inducing a pregnancy and yielding offspring following transfer (Gandolfi *et al.* 1998). The incidence of blastocyst formation is often used as an indicator of oocyte competence in the laboratory (Gandolfi *et al.* 1998). However, blastocyst development from an oocyte does not guarantee development to term. For example, polyspermic porcine zygotes are still able to form blastocyst stage embryos but have poor viability, with greater than 78 % displaying abnormal ploidy and compromised development (Han *et al.* 1999a; McCauley *et al.* 2003). Other indicators of embryo viability include metabolism, allocation and number of cells in blastocysts, ability to survive freezing,

blastocyst morphology, developmental kinetics, chromosomal segregation, gene expression, protein profile, degree of apoptosis, pregnancy statistics and development to term (reviewed in Krisher *et al.* 1999; Kidson 2005). A number of follicle and oocyte parameters (e.g. size, composition) also indicate subsequent developmental competence and these will be discussed in dedicated sections later in this review.

At fertilisation, sperm penetration initiates oscillations in the concentration of intracellular free calcium, which in turn triggers cortical granule release to prevent polyspermy, resumption and completion of meiosis, formation of the male pronucleus and subsequent mitotic cleavage (reviewed by Schultz and Kopf 1995). Parthenogenetic activation induces oscillations in the concentration of intracellular-free calcium in the absence of sperm using a number of different physical and/or chemical treatments. The main methods include application of electrical pulses, injection of ethanol, and exposure to inositol triphosphate agonists, thimerosal, protein synthesis inhibitors, sperm cytosolic factors, calcium ionophores, and protein kinase inhibitors (Ozil 1990; Fukui *et al.* 1992; Loi *et al.* 1998; Machaty *et al.* 1998). As mentioned in the introduction, embryo development following pig embryo IVP is hampered by a high incidence of polyspermy and a low rate of male pronuclear development (reviewed by Niwa 1993 and Nagai 1994). Parthenogenetic activation can be used to overcome the problems associated with fertilisation systems and reduces the variability of embryo production (O'Brien *et al.* 2000; Marchal *et al.* 2001). Parthenogenetic embryos are able to develop to the blastocyst stage *in vitro* and following transfer into a recipient animal. Zhu *et al.* (2003) reported that there was no significant difference in rate of blastocyst development following parthenogenetic activation of *in vitro* and *in vivo* matured pig oocytes (61% vs. 46% respectively, $p > 0.05$). Pig parthenogenetic fetuses appear morphologically normal, but are smaller and lighter than those derived from IVM-IVF

and a developmental block around 31 days post oestrus has been reported (Zhu *et al.* 2003). Parthenogenetic blastocyst development followed by embryonic failure during implantation has also been reported in the cow, sheep, mouse and rabbit (Kaufman *et al.* 1977; Ozil 1990; Fukui *et al.* 1992; Loi *et al.* 1998). This suggests that parthenogenetic activation is a reliable tool for examining the early stages of embryo development in the pig.

1.6 Factors effecting maturation and developmental competence in the pig

1.6.1 Donor age

The use of pre-pubertal animals as oocyte donors for domestic species embryo IVP can shorten the generation interval and thus increase the rate of genetic gain achievable through conventional reproductive or embryo manipulation technologies (Georges and Massey 1991; Lohuis 1995). Pre-pubertal animals are generally preferred for meat production and hence are the most common source of oocytes used in IVM and related reproductive technologies. Offspring have been produced following *in vitro* maturation and fertilisation of oocytes from pre-pubertal cows (Armstrong et al. 1992; Armstrong et al. 1994a), sheep (Armstrong et al. 1994b; O'Brien et al. 1996), goats (Mogas et al. 1995) and pigs (Yoshida *et al.* 1990; Yoshida *et al.* 1992; Marchal *et al.* 2001). In pigs, *in vitro* matured pre-pubertal oocytes have even been used to produce nuclear transfer embryos that have developed to term following transfer to recipient pigs (Lai et al. 2002; Park et al. 2002).

Despite these successes, oocytes sourced from pre-pubertal donors display reduced developmental competence when compared to adult oocytes following embryo IVP procedures (reviewed by Armstrong 2001). Embryo IVP from pre-pubertal oocytes is characterised by low rates of blastocyst formation in the cow (Revel *et al.* 1995; Khatir

et al. 1996; Presicce *et al.* 1997; Taneja *et al.* 2000; Oropeza *et al.* 2004), sheep (O'Brien *et al.* 1996; Morton *et al.* 2005), goat (Mogas *et al.* 1997) and pig (see Table 1.2) (Pinkert *et al.* 1989; O'Brien *et al.* 2000; Marchal *et al.* 2001; Grupen *et al.* 2003; Ikeda and Takahashi 2003; Sherrer *et al.* 2004). Even *in vivo* derived oocytes from pigs in first oestrus are less developmentally competent than those at their third oestrus (Archibong et al. 1987; Menino et al. 1989). Nuclear and cytoplasmic transfer studies in both pig and cow suggest that the low developmental ability of pre-pubertal oocytes relates to incomplete cytoplasmic maturation and/or incomplete growth (Kuhholzer *et al.* 2001; Salamone *et al.* 2001).

Oocytes appear to have obtained the factors they need to complete meiosis by the antral follicle stage, but do not acquire the factors required for complete cytoplasmic maturation and early embryo development until the final stages of pre-ovulatory follicle growth. As such, the population of follicle sizes used for IVM may contribute to quality variation, with pre-pubertal ovaries probably containing smaller follicles than adult on average. In sheep, oocyte diameter and meiotic competence (to MII) of pre-pubertal and adult oocytes increased with follicle size from <1 mm to > 2 mm (Ledda *et al.* 1999). In both pre-pubertal and adult goat oocytes, the rate of maturation, oocyte diameter, and number of cumulus layers increases in a similar and stepwise manner with follicle size (Martino *et al.* 1994). In cattle, the developmental competence of pre-pubertal and adult oocytes increases with follicle size from 2-3 mm to 4-8 mm to >8 mm (Kauffold *et al.* 2005). These findings suggest that since the follicles of pre-pubertal animals are still growing and developing, a greater proportion of smaller follicle sizes may occupy the IVM aspiration range and contribute to the low developmental competence of pre-pubertal oocytes.

Despite developmental variation, few differences appear to exist in terms of final nuclear maturation between pre-pubertal and adult oocytes. Despite extensive testing in a number of species (see Table 1.2 and review by (reviewed by Armstrong 2001), only two bovine studies report a higher rate of nuclear maturation of cow compared to calf oocytes during IVM (Khatir *et al.* 1997; Gandolfi *et al.* 1998). The kinetics of nuclear maturation between pre-pubertal and adult pig oocytes still need to be compared. The fact that a number of the cell cycle molecules that regulate meiosis are cytoplasmic factors also suggests that abnormal/incomplete meiotic maturation in pre-pubertal oocytes could result from cytoplasmic deficiencies.

In cattle, pre-pubertal oocytes differ from adult oocytes in terms of ultrastructure, including abnormal chromatin and microtubule configurations (Damiani *et al.* 1996; de Paz *et al.* 2001), biochemical features; reduced levels (Damiani *et al.* 1998; Salamone *et al.* 2001) and activity (Salamone *et al.* 2001) of Histone H1-kinase (indicative of MPF activity) and MAPK and lower amount of inositol 1,4,5-trisphosphate receptor (IP(3)R), metabolic activity; reduced amino acid uptake, reduced metabolism of glucose, glutamine and pyruvate (Gandolfi *et al.* 1998; Steeves and Gardner 1999) and molecular activity, different patterns of mRNA and protein (Levesque and Sirard 1994; Khatir *et al.* 1996; Gandolfi *et al.* 1998; Khatir *et al.* 1998), and aberrant calcium oscillation post fertilisation (Damiani *et al.* 1996; Damiani *et al.* 1998). There is not a great deal known about specific cytoplasmic differences between pre-pubertal and adult pig oocytes, so far they appear to have similar ultrastructure, metabolic characteristics and MPF activity (O'Brien *et al.* 2000; Ikeda and Takahashi 2003). However, glutathione synthesis has been shown to increase in oocytes from first to multiple oestrous in pigs (Herrick *et al.* 2003) and in adult compared to pre-pubertal donors (de Matos *et al.* 2003). In sheep, differences in ultrastructure; smaller mitochondria and cortical granules (O'Brien *et al.*

1996), communication; fewer transzonal projections and reduced methionine uptake (Ledda *et al.* 2001), metabolism; reduced glutamine uptake (O'Brien *et al.* 1996), and reduced MPF activity (Ledda *et al.* 2001) have also been reported for pre-pubertal versus adult oocytes.

Table 1.2 Maturation, fertilisation and embryo development of oocytes sourced from pre-pubertal and adult pigs

		% Oocytes				
Reference	Donor Age	Maturation	Fertilisation	Cleavage	Blastocyst	Total Cell No.
O'Brien et al. 2000	Pre-pubertal	79	82 [a]			
	Adult	92	54 [b]			
Marchal et al. 2001	Pre-pubertal	85 [a]	52 [a]	34 [a]	29 [b] [10 [a]][1]	
	Adult	83 [a]	46 [a]	38 [a]	14 [a] [36 [c]][1]	
Grupen et al. 2003	Pre-pubertal		Parthenogenetic	73 [a]	38 [a]	30 [a]
	Adult		activation	92 [b]	57 [b]	43 [b]
Ikeda and Takahashi 2003	Pre-pubertal	≈ 93 [a]	Parthenogenetic	62 [a]	32 [a]	30 [a]
	Adult	≈ 100 [a]	activation	79 [b]	45 [a]	30 [a]
Sherrer et al. 2004	Pre-pubertal	≈ 88 [a]	62 [a]	≈ 52 [a]	≈ 2 [a]	≈ 29 [a]
	Adult	≈ 90 [a]	57 [a]	≈ 60 [a]	≈ 14 [b]	≈ 36 [b]

≈ Author measurement from published graphs

[1] Blastocyst per total
[ab] Different superscripts within a parameter and study indicate significant difference (P>0.05)

1.6.2 Follicle and oocyte size

While mouse oocytes reach their full size by the antral follicle stage (Sorensen and Wassarman 1976), oocytes of large domestic animals, such as the pig, continue to grow until the late antral follicle stage (Motlik *et al.* 1984). During the growth phase in a wide range of species, oocyte diameter increases along with the ability to complete meiotic maturation (Sorensen and Wassarman 1976; Moor and Trounson 1977; Motlik and Fulka 1986; Schramm *et al.* 1993; Fair *et al.* 1995; Otoi *et al.* 1997; Hewitt and England 1998) and the ability to undergo successful fertilisation and embryo development (Moor and Trounson 1977; Otoi *et al.* 1997; Lucas *et al.* 2003). There is a close positive relationship between oocyte size and increasing follicle size until full growth is attained, in a wide variety of species including the pig (Griffin *et al.* 2006). Meiotic and early embryo development are closely related to increasing follicle size in a number of species including the cow (Pavlok *et al.* 1992; Lonergan *et al.* 1994; Torner *et al.* 2001; Kauffold *et al.* 2005; Lequarre *et al.* 2005), sheep (Moor and Trounson 1977; Ledda *et al.* 1999) and pig (Yoon *et al.* 2000; Sun *et al.* 2001; Lucas *et al.* 2002; Marchal *et al.* 2002; Lucas *et al.* 2003). In most species, oocytes from follicles < 3 mm in size are not fully meiotically competent, resulting in low rates of MII following IVM, and have extremely limited ability to support embryo development following IVM (reviewed by Abeydeera 2002). Pig oocytes from follicles > 3 mm reach MII, cleave and form blastocyst embryos at a significantly higher rate than those from follicles < 3 mm; and this is why oocytes are aspirated from follicles 3-8 mm in size (Motlik and Fulka 1986; Bolamba and Sirard 2000; Yoon *et al.* 2000; Sun *et al.* 2001; Liu *et al.* 2002; Lucas *et al.* 2002; Marchal *et al.* 2002; Ikeda and Takahashi 2003). Table 1.3 illustrates the studies that have examined oocyte meiotic or developmental competence in pig oocytes from different follicle sizes. The findings of these studies suggest that as the follicle

increases in size, important changes occur in cumulus cell and oocyte derived factors required to support oocyte maturation.

As discussed earlier, cumulus cells secrete and communicate factors to the oocyte that are essential for both nuclear and cytoplasmic maturation. IVM of denuded pig oocytes (DOs) is incompatible with blastocyst development, and while DOs from follicles 3-6 mm can still complete meiosis to MII and cortical granule migration, DOs from follicles 0.5-2 mm are incapable of either of these processes (Sun *et al.* 2001). Cytoplasmic processes, MAPK phosphorylation and cyclin B1 synthesis were missing in denuded oocytes from follicles 1-2 mm in diameter compared to those from 3-6 mm follicles. In COCs isolated from 6-7 mm follicles, treatment with EGF can stimulate hyaluronic acid production, F-actin rearrangement and cumulus expansion, but has no effect on COCs isolated from follicles smaller than 4 mm in the pig. A higher degree of cumulus expansion was also observed for pig COCs from 7-8 mm follicles (63%), compared to those from 3-6 mm (7%) and 1-2 mm (0%) follicles upon collection for IVM (Liu *et al.* 2002). This correlated to an extent with number of layers of cumulus cells at the time of collection, with a greater percentage of COCs from 3-6 mm (65%) and 7-8 mm (45%) follicles displaying more than 4 cumulus layers compared to 1-2 mm follicles (18%) (Liu *et al.* 2002). Co-culture of follicle-enclosed oocytes with cumulus cells from follicles larger than 3 mm confers an increased rate of follicle and oocyte growth and survival in culture, when compared to co-culture with cumulus cells isolated from follicles smaller than 3 mm (Wu *et al.* 2002). Oocytes from smaller follicles therefore appear to have reduced cumulus expansion, reduced cumulus layers and are more adversely affected by removal of the cumulus layer than oocytes from larger follicles.

The effect of follicle size on a number of factors that are secreted by or act via cumulus cells to affect oocyte maturation has also been studied. Cumulus and granulosa cells

from 1-2 mm porcine follicles are less responsive to LH and hCG stimulation, secreting less progesterone in culture than cumulus cells from 3-5 mm and 6-12 mm follicles (Channing *et al.* 1981). Different steroidogenic responses have been observed in porcine granulosa cells from < 3 mm and 3-6 mm follicles in response to a wide range of modulators including forskolin, cAMP analogue, protein kinase-c activator, FSH, and calcium ionophore (Hylka *et al.* 1989; Hylka and diZerega 1990). Concentrations of 17β-oestradiol increase more than 7 fold in porcine FF from 1-2 and 3-6 mm follicles to FF from 7-8 mm follicles, while progesterone concentrations increase 1.5 fold from FF of 1-2 mm follicles to FF from 3-6 and 7-8 mm follicles (Liu *et al.* 2002). progesterone and 17β-oestradiol concentrations also increase from 8-13 to > 14 mm follicles in human FF (Teissier *et al.* 2000). This does not seem to be the case in the cow, with FF from < 6, 6-8 and >9 mm follicles demonstrating similar effects on oocyte cytoplasmic maturation (Carolan *et al.* 1996).

While the exact relevance of steroid levels in FF to oocyte maturation may not be completely defined, the beneficial effect of FF during IVM on subsequent blastocyst formation is well acknowledged. IVM supplementation with porcine FF from 3.1-8 mm follicles compared to < 3.1 mm follicles increases the blastocyst development of oocytes from follicles < 3.1 mm and > 3.1 mm (Yoon *et al.* 2000). In adult pigs (as opposed to the usual studies in pre-pubertal animals), cumulus expansion, maturation, cleavage and blastocyst development of COCs was higher following maturation with FF from 5-8 mm follicles, compared to 2-4 mm follicles. The use of FF from small follicles has been shown to have an inhibitory effect on cumulus expansion during IVM, and this has been shown to decrease with the use of FF from follicles of increasing size (< 2 mm to 2-5 mm to > 5 mm) (Qian *et al.* 2003).

A number of cytoplasmic factors also appear to be acquired with increasing follicle and/ or oocyte size. Bovine studies have revealed that oocytes from small follicles display deficient mRNA and protein accumulation (Pavlok *et al.* 1992; Lonergan *et al.* 1994). In the pig, a correlation has been reported between reduced oocyte diameter and decreased oocyte GSH content before IVM, suggesting that small oocytes have not acquired all the factors/ molecules required for subsequent cytoplasmic maturation (Liu *et al.* 2002). Pig oocytes from 4-6 mm follicles, in contrast to those from 0.5-0.7 mm follicles, have the ability to activate cell cycle molecules Cdc2 kinase and MAP-kinase (Kanayama *et al.* 2002). Similarly, the level of cyclin B is higher in oocytes from follicles >3 mm, compared to 1-3 mm (Wu *et al.* 2006). P34cdc2 and cyclin B expression does not appear to change in oocytes and cumulus cells from <2 mm, 3-5 mm and > 6 mm follicles in the cow (Robert *et al.* 2002). Expression of three developmental genes (DNA-methyltransferase I, Pou domain class 5 transcription factor 1 and FGF receptor 2) increases in oocytes from >3 mm compared to 1-3 mm follicles (Wu *et al.* 2006). In the goat, oocytes from meiotically competent follicles (> 3 mm), display nucleolar compaction and decreased RNA synthesis compared to smaller follicle categories ranging from 0.5 to 3 mm (de Smedt *et al.* 1994). Only slight differences in protein patterns in oocytes from different follicle size categories have been reported in pig, cow and goat studies (de Smedt *et al.* 1994; Marchal *et al.* 2002; Lequarre *et al.* 2005; Wu *et al.* 2006). Considering its importance to both meiotic and developmental competence, it is surprising that the intra-oocyte cAMP content has not been examined in the different follicle sizes.

Table 1.3 Size, maturation, fertilisation and embryo development of oocytes sourced from different sized follicles in the pig

	Size		% Oocytes		
Reference	Follicle (mm)	Oocyte (µm)	MII	Fertilisation Parameter*	Blastocyst
Motlik & Fulka, 1986	0.3-0.7	100	0		
	1.8-2.2	115	49		
	5.0-6.0	120	76		
Yoon et al. 2000	< 3.1		58	81	2
	3.1-8		91	90	10
Sun et al. 2001	0.5-2		35 [a]	56 [1a]	5 [a]
	3-6		64 [b]	97 [1b]	23 [b]
Marchal et al. 2002	< 3		44 [a]	53 [a]	3 [a]
	3-5		77 [b]	73 [b]	14 [b]
	>5		86 [b]	77 [b]	23 [b]
Liu et al. 2002	1-2	120 [a]	≈ 50 [a]		
	3-6	128 [b]	≈ 75 [b]		
	7-8	138 [c]	≈ 82 [b]		
Lucas et al. 2002	0.4-0.99	129 [a]			
	1.0-2.19	137 [b]			
	2.2-2.79	141 [c]			
	2.8-3.59	144 [d]			
	3.6-6.5	145 [d]			
Lucas et al. 2003	0.4-0.99			50 [a]	
	1.0-2.19			70 [a]	
	2.2-2.79			84 [b]	
	2.8-6.5			86 [b]	
		<105		67 [x]	
		105-109		64 [x]	
		110-114		76 [y]	
		>115		77 [y]	

* Penetration/MPN
[abxy] Differences between superscripts [ab] or [xy] represent significant differences
[1]CG migration represented as a fertilisation parameter
≈ Author measurement from published graphs

1.7 Summary

The precise factors and conditions that yield a good quality oocyte following IVM are poorly understood. Appropriate co-ordination of both nuclear and cytoplasmic maturation during IVM appears necessary, given that use of agents, such as dbcAMP, to synchronise meiotic maturation during IVM improve subsequent blastocyst development. There is limited information concerning the levels of intra-oocyte cAMP during IVM and, in particular, variation in intra-oocyte cAMP between groups of oocytes with different developmental competencies. IVM oocytes from pre-pubertal versus adult donors in the pig provide a model of high versus low developmental competence. Similarly, the maturational and developmental competence of oocytes improves with increasing follicle size, but how strong this relationship holds within the aspiration range employed for IVM is not well understood. The contribution of follicle size to the final developmental competence of oocytes from pre-pubertal and adult donors has not been defined. The literature reviewed in this chapter highlights the absence of studies examining the effect of donor age and follicle size on intra-oocyte cAMP levels during IVM and the impact that these levels have on subsequent developmental competence.

1.8 Thesis hypothesis and aims

1.8.1 General hypothesis

High intra-oocyte cAMP concentration during oocyte maturation in vitro improves subsequent oocyte developmental competence by increasing co-ordination between the processes involved in nuclear and cytoplasmic maturation.

1.8.2 Specific hypotheses

In order to test the general hypothesis it was necessary to understand the conditions resulting in high and low developmental competence in pre-pubertal and adult oocytes. Thus, it was hypothesized that:

1. Increased oocyte developmental competence is associated with changes in ovarian morphology that occurs immediately following the onset of puberty in the pig.
2. Porcine oocyte developmental competence increases with increasing follicle size in adult and pre-pubertal donors and is a function of the distribution of follicle size cohorts.
3. The ability to increase intra-oocyte cAMP concentration during IVM is correlated with oocyte developmental competence.
4. Dibutyryl cAMP mediates its positive effect on oocytes by increasing the intra-oocyte cAMP during IVM.
5. Increased intra-oocyte cAMP during IVM is a result of extended GJC between the cumulus cells and the oocyte.

To test these specific hypotheses the aims of this study were to:

1. Determine ovarian, follicular and oocyte morphology and developmental competence at the onset of puberty in the pig.
2. Examine the response of pre-pubertal and adult pig oocytes to dbcAMP exposure for the first 22 h of IVM.
3. Compare the proportion, steroidal composition and subsequent oocyte developmental competence between 3, 4 and 5-8 mm follicles of pre-pubertal and adult ovaries.

4. Determine the effect of dbcAMP for the first 22h IVM on the developmental competence of pre-pubertal oocytes from 3, 4 and 5-8 mm follicles.
5. Determine the cAMP content and cumulus expansion of, and examine the effect of dbcAMP treatment on, pre-pubertal oocytes and COCs from 3 mm and 5-8 mm follicles.
6. Assess the effect of dbcAMP treatment on cumulus-oocyte GJC in pre-pubertal COCs from 3 mm and 5-8 mm follicles.

Chapter 2

Methods and Materials

2 Methods and materials

2.1 Chemicals and media

All chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. All media, buffers and solutions were prepared using milli-Q water (Millipore, Bedford, MA, USA). Pre- IVM washing was conducted in HEPES-buffered tissue culture medium-199 (H-TCM; with Earle's salts, L-glutamine, 2.2 mg/ml sodium bicarbonate and 25 mM Hepes Buffer) supplemented with 25% (v/v) porcine FF. The basic IVM medium was bicarbonate buffered tissue culture medium-199 (B-TCM; with Earle's salts, L-glutamine, 2.2 mg/ml sodium bicarbonate) supplemented with 0.1 mg/ml sodium pyruvate, 75 μg/ml penicillin-G, 50 μg/ml streptomycin sulfate, 20 μg/ml porcine FSH (Follitropin-V, Vetrepharm Canada, Inc, London Canada) (equivalent to 60 mIU recombinant human FSH (rhFSH)/ml), 1.0 μg/ml oestradiol-17β, 0.5 mM cysteamine, 10 ng/ml EGF and 25% porcine FF. For the majority of experiments, H-TCM and B-TCM were Invitrogen brand, purchased from Gibco-BRL (Grand Island, NY, USA), except for in Chapter 3 (Invitrogen brand, Gibco-BRL, Milan, Italy) and Chapter 4 (ICN Biomedicals, CA, USA). In addition to control media, separate H-TCM and B-TCM supplemented with 1mM dbcAMP was also prepared.

FF for media supplementation was prepared from a pool of follicular aspirates from pre-pubertal ovaries by centrifugation (2,000g for 30 min) of the material aspirated from antral follicles of 3-8 mm in diameter, filtered though a sterile 0.22 μm pore filter (Millipore) and stored at –80°C. Pre-activation/IVF washing and polar-body searching was conducted in phosphate buffered North Carolina State University-23 medium (PB-NCSU-23; (Petters and Wells 1993) supplemented with 4.0 mg/ml bovine serum

albumin (BSA; ICP Bio, New Zealand). For IVF, Sperm Pre-incubation Medium (SPM199) consisted of B-TCM supplemented with 0.91 mM Sodium Pyruvate, 2.92 mM Calcium Lactate, 1.0 mM L-glutamine, 75 mg/L penicillin-G, 50 mg/L streptomycin sulfate, 0.4% heat inactivated-fetal calf serum (FCS; Invitrogen, Gibco-BRL, Grand Island, NY, USA). Activation with ionomycin and fertilisation procedures were carried out in tyrode-albumin-lactate-pyruvate-poly-vinyl alcohol (TALP-PVA) medium (Bavister 1989) supplemented with 3.0 mM calcium lactate and 2.0 mM caffeine-sodium benzoate. Activation with 6-dimethylaminopurine (6-DMAP) and subsequent IVC was conducted in NCSU-23 medium (Petters and Wells 1993) supplemented with 4.0 mg/ml BSA. All media used for culture was equilibrated for a minimum of 3 h prior to use at 38.5^{o}C in a humidified atmosphere of 5% CO_2 in air.

2.2 *In vitro* oocyte maturation

Ovaries were collected from slaughtered pre-pubertal and adult Large White/Landrace cross bred pigs and transported to the laboratory in saline (0.9% w/v NaCl; Baxter Healthcare Corporation, Deerfield, IL, USA) supplemented with antibiotics and maintained at 38°C (50 IU/ml penicillin G and 50 μg/ml streptomycin sulfate, CSL Bioscience, Parkville, Vic, Australia). Upon arrival, the ovaries were washed thoroughly in 38.5°C pre-warmed saline solution and were kept at 38.5°C during processing. The research described in Chapter 3 was conducted in Milan, Italy while the experiments in Chapters 4-7 were conducted in Adelaide, Australia. Where possible the same materials and suppliers were employed, but please refer to the Bagg *et al.,* 2004 publication in Appendix 1 for additional information. Ovaries from different donor age groups were mostly collected on separate days due to slaughterhouse schedules, with time from ovary collection to start of maturation kept constant at approximately 3 h. Follicle aspiration was conducted with a 21-gauge needle, though which constant

suction (1 L/min) was applied. Follicular contents were aspirated from antral follicles (3-8 mm in diameter) and pooled in a collection tube. COCs with at least three intact, compact layers of cumulus cells were recovered from the collected FF and washed three times in H-TCM. Before transfer to treatments, COCs were washed two times in dishes containing B-TCM, with or without 1mM dibutyryl cAMP (dbcAMP) as appropriate. Groups of 15-20 COCs were transferred to 50 µl droplets of B-TCM in a 35 mm Falcon culture dish (Becton Dickinson and Company, Franklin Lakes, NJ, USA), with or without dbcAMP as appropriate, and incubated at 38.5^{o}C in a humidified atmosphere of 5% CO_2 in air. Following 22 h IVM, COCs were washed and transferred to 50 µl droplets of B-TCM without dbcAMP, and incubated for another 22 to 24 h, depending on the experiment. Embryo tested mineral oil was used to cover all media droplets.

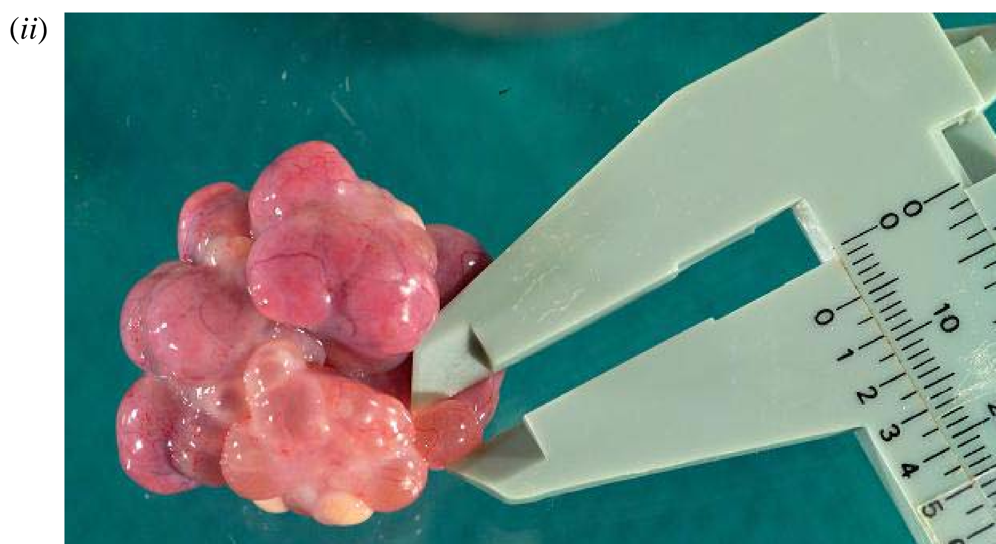
2.3 Measurements

Stringent and consistent methods were employed when ovary, follicle and oocyte measurements were required. Ovaries were measured using a sterile ruler with mm increments. Follicles were individually measured with callipers (Figure 2.1), with size group cohorts kept as close to the named size as possible, follicles with diameters smaller or larger than 3 mm above whole numbers excluded from the study to ensure there was adequate distinction between the follicle size cohorts. For example, for the 4 mm follicles, any diameters of 4.3 mm and above or 2.7 mm and below were discarded. The diameters, both with and without the zonae pellucida, of randomly selected intact oocytes following 48 h IVM were measured using a stereomicroscope with graticules corresponding to micrometers (µm) at 6.3 x magnification.

Figure 2.1. Measurement of individual follicles on pre-pubertal (*i*) and adult (*ii*) with mm increment callipers.

(*i*)

(*ii*)



2.4 Cumulus cell removal

Two different methods of cumulus cell removal were employed, depending on whether the denuded oocytes were required for embryo IVP and the GJC assay, or for the purpose of nuclear maturation staining and cAMP analysis. Droplets containing oocytes for embryo IVP were treated with 1 mg/ml hyaluronidase in phosphate buffered saline (PBS) supplemented with 5% FCS for 1 min at the end of IVM and aspirated in PB-NCSU-23 with a small-bore glass pipette to gently remove cumulus cells. For the GJC assay, hyaluronidase was added to the well of H-TCM, with or without dbcAMP treatment, that contained the COCs for denuding to give a final concentration of 1 mg/ml. COCs were incubated in 1 mg/ml hyalronidase for 3 minutes then aspirated with a small-bore glass pipette to gently remove cumulus cells. When the oocytes were required for nuclear maturation staining and cAMP analysis, cumulus cells were removed by vortexing for 4 min in a 10 ml tube (Falcon) containing 2 ml H-TCM.

2.5 Embryo production

Only matured oocytes, displaying a polar body, were selected for parthenogenetic activation and IVF methods of embryo production.

2.5.1 Parthenogenetic activation

The 6-DMAP was dissolved in NCSU-23 medium and equilibrated for 3 h prior to use. Ionomycin, stored as a stock solution of 5 mM in 100% ethanol at -20C, was diluted immediately prior to use in equilibrated TALP-PVA medium to give a working concentration of 5 μM. Groups of oocytes were sequentially incubated in 5 μM ionomycin for 5 min and then 2.0 mM 6-DMAP for 3 h.

2.5.2 Fertilisation

For each experimental replicate, a single commercial dose (80 ml) of fresh Large White boar semen, extended in Androhep (Minitüb, Landshut, Germany), was purchased from a local boar testing facility (Sabor LTD, Clare, Australia). After transport and storage (48 h maximum) at room temperature (21°C), the semen was centrifuged (5 min, 300g) and washed twice by re-suspension in SPM199 and centrifugation (5 min, 300g). The sperm pellet was then resuspended in 10 ml pre-equilibrated SPM199 and incubated for 45 min in a humidified atmosphere of 5% CO_2 in air. Following incubation of the sperm suspension, supernatant was removed and the pellet diluted in TALP-PVA to 5 x 10^6 cells / ml. Oocytes were washed once and transferred to TALP-PVA insemination drops (90 μl) overlaid with mineral oil (maximum of 25-30 oocytes per drop). Oocytes were spaced equally from one another around the edge of the insemination drop. A 10 μl aliquot of sperm containing media from the top of the tube (swim-up method) was added to the centre of each insemination drop to give a final dilution of 5 x 10^5 sperm/ml. After 10-20 min co-culture the oocytes and zona-attached sperm were gently transferred to fresh 100 μl drops of TALP-PVA media covered with mineral oil and co-cultured for 8 h at 38.5^{o}C in a humidified atmosphere of 6% CO_2 in air. Following insemination, presumptive zygotes were aspirated with a small-bore glass pipette to remove any remaining cumulus cells and zona attached sperm.

2.5.3 Embryo culture

Following either parthenogenetic activation or IVF, presumptive zygotes were washed twice and transferred to 50 μl droplets (15-20 presumptive zygotes per drop) of NCSU-23 culture medium, containing 4.0 mg/ml BSA, and incubated at 38.5^{o}C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2. After 5 days of IVC, embryonic cleavage

was assessed and 10% FCS was added to the culture drops. After 7 days of IVC, embryonic cleavage and blastocyst development was assessed.

2.6 Steroid content assessment

Samples of spent IVM media were stored at –20°C and pooled FF from 3 mm, 4 mm and 5 mm follicles were stored at –80 ^{o}C, in preparation for steroid analysis. Concentrations of progesterone and 17β-oestradiol were determined in the various samples using commercially available diagnostic radioimmunoassay kits (Diagnostic Systems Laboratories, Webster, TX, USA). Standard curves were verified by serial dilution of a representative sample of media to establish the appropriate dilutions. The assay limits of detection were 0.4 and 0.016 nmol/L for progesterone and 17β-oestradiol respectively. The intra- and inter-assay coefficients of variation were 8.9 and 11.7% for progesterone and 4.2 and 13.1% for 17β-oestradiol, respectively. All samples were analysed in a single assay to avoid inter-assay variation.

2.7 Intra-oocyte cAMP assay

The cAMP content of zona-free oocytes was measured using a radioimmunoassay method described and validated previously (Reddoch et al. 1986). At 0 and 22 h IVM oocytes were denuded by vortex as described previously, and washed twice in PBS containing 10% FCS. Oocytes were treated with 0.5% pronase in H-TCM for 10-20 sec to remove the zonae pellucida and then washed 5 times in PBS containing 10% FCS. Upon comparing denuded and zona-free oocytes (refer to Appendix 2), we supported previous findings that zonae pellucida removal is important in the detection of intra-oocyte cAMP, eliminating any possible influence of adenylate cyclase present in the remains of the corona cell foot processes, many of which remain embedded in the zonae pellucida after denuding (Mattioli *et al.* 1994). Twenty to 30 oocytes were stored in

500µl ethanol at –20°C in preparation for assaying. A sample of each final wash was also collected to ensure complete washing of oocytes that were matured in dbcAMP. Samples were vortexed for 30 sec and pulse centrifuged before being evaporated by passing N_2 gas over the surface of the solution within the tube held in a heat block set at 37°C. The cAMP-containing residue remaining in the tube was then re-suspended in 220 µl assay buffer (50 mM sodium acetate, pH 5.5). The samples were then acetylated with 6.67 µl of triethylamine [AJAX Chemicals, Sydney, Australia]: acetic anhydride [BDH Laboratory supplies, Poole, England] (2:1 v/v) and 100 µl duplicates were aliquoted for the assay. ^{125}I-labelled cAMP with specific activity of 2010 Ci/mM (prepared by iodinating 2'-0-monosuccinyladenosine-3':5'-cyclic monophosphate tyrosyl methyl ester using the chloramine T method; (Hunter 1970) and cAMP antibody (Reddoch et al. 1986) were added to duplicate samples and incubated for 24 h at 4°C. To precipitate, 1 ml of 100% ethanol at 4°C was then added to the sample. Following centrifugation at 3000 g for 10 min, the supernatant was decanted and the pellet was dried in air and counted with a gamma counter. Triplicate standards were used to produce a standard curve (0-1000 fmol cAMP). The cross reactivity between dbcAMP and the cAMP antibody employed in this assay was around 1% (Appendix 3), suggesting that these results represent the actual cAMP content of the COC. In addition, the low values observed at 0 h for dbcAMP treated oocytes provides confidence that there is minimum residual cAMP being measured.

2.8 Assessment of cumulus expansion

Cumulus expansion of COCs was recorded after 22 h or 46 h IVM. The degree of cumulus expansion was assessed according to a subjective scoring system (0 to 4). Essentially, a score of 0 indicates no expansion and a score of 4 indicates complete

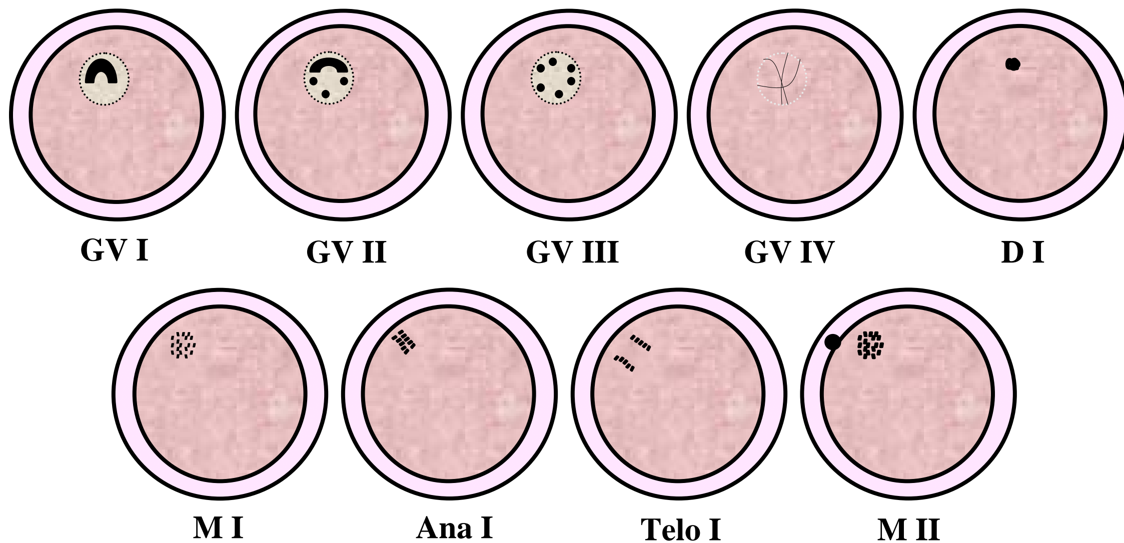
cumulus expansion in all cumulus layers. The cumulus expansion index (0.0 – 4.0) was calculated as previously described (Fagbohun and Downs 1990; Vanderhyden et al. 1990).

2.9 Staining

2.9.1 Orcein staining of oocytes

Denuded oocytes were fixed in a solution of ethanol and acetic acid (3:1; v/v) for 48 h in a 4-well dish (Nunc International), mounted on a microscope slide underneath a cover slip and stained with 1% Orcein in 45% (v/v) acetic acid. Oocytes were de-stained with a solution of glycerol, acetic acid and water (1:1:3 v/v). Oocytes were examined under a phase contrast microscope at 400X magnification (Leitz Labourlux-S, Wetzlar, Germany) and meiotic stages were classified based on previously described criteria (Motlik and Fulka 1976; Homa 1988). Figure 2.2 depicts the stages of meiosis during spontaneous porcine oocyte maturation as observed following orcein staining. The stages of meiotic progression were assessed as germinal vesicle (GV) (stages I-IV), MI (diakinesis I (D I) - MI) and MII (anaphase I (ana I), telophase I (telo I) and MII).

Figure 2.2 The stages of meiosis during spontaneous porcine oocyte maturation as observed following orcein staining. Original artwork based on author observations and description in text by Motlik and Fulka (1976).



2.9.2 Hoechst staining of blastocysts

Blastocysts were treated with a 0.5% pronase solution to dissolve the zonae, washed in H199 and incubated for 30 min in 0.05 mM bisbenzamide (Hoechst 33342). Blastocysts were rinsed in 100% ethanol and mounted on a microscope slide in glycerol beneath a coverslip. Nuclei were visualised and counted using fluorescence microscopy (Olympus AH3 microscope, Olympus, Tokyo, Japan).

2.10 GJC assay

The GJC between the oocyte and the cumulus cell layer of pre-pubertal COCs from 3 mm or >5 mm follicles was measured using a previously described and validated method (Thomas *et al.* 2004) of quantitative fluorescence microscopy that utilises the acetoxymethyl (AM) ester derivative of the fluorescent indicator calcein (calcein-AM; 3’, 6’-Di(O-acetyl)-2’,7’-bis[N,N-bis(carboxymethyl) amino methyl]-fluorescein,

tetraacetoxy methyl ester; C-3100; Molecular Probes; Eugene, OR, USA). Calcein-AM is a non fluorescent, electrically neutral and highly lipophilic molecule with high cell membrane permeability, meaning it can easily enter the oocyte cytoplasm (Wang *et al.* 1993; Lichtenfels *et al.* 1994). Once inside the cell, non-specific endogenous esterases cleave the lipophilic acetoxymethyl groups leaving calcein, a highly fluorescent, negatively charged molecule that cannot leak out of cells via the plasma membrane, but can pass between cells via intact gap junctions. Cumulus-oocyte GJC can be measured using fluorescence microscopy to quantify the amount of calcein transferred from the cumulus cells into oocyte via the gap junctions.

Vials of calcein-AM (50 μg) were stored desiccated at –20°C and reconstituted with 100μl DMSO to prepare a 0.5 mM calcein-AM stock solution for each experiment. A new vial of calcein-AM was used at each time-point in each experiment to make fresh calcein-AM stock, since the calcein-AM in solution can hydrolyse over time to generate fluorescent calcein. At the concentrations of calcein-AM used, the final concentration of DMSO was <0.01%. COCs were cultured in B-TCM as described above, with or without dbcAMP, for 0, 6, 11 and 22 h after which they were transferred to phenol red free H-TCM (PRF H-TCM) with or without dbcAMP in a 46 well plate (Falcon). The timing for all washes was kept constant at exactly 1 min and 30 sec. After two washes in PRF H-TCM with or without dbcAMP, oocytes were transferred in exactly 50 μl PRF H-TCM with or without dbcAMP to 450 μl of 1 μM calcein-AM freshly made up in PRF H-TCM with or without dbcAMP with 0.2 mg/ml PVA for exactly 5 min. It was confirmed, using confocal analysis, from COCs incubated in calcein-AM for 2, 5, 10 or 15 min, that the 5 min incubation gives the best result with dye in all layers of the COC but not inside the oocyte. Following the calcein-AM incubation, COCs were then washed twice in PRF H-TCM with or without dbcAMP and then cultured for a further

25 min to enable dye exchange between cumulus layer and the oocyte. Hyaluronidase was added to the well 3 min before the end of incubation and then the oocyte was denuded using the mouth pipette method outlined above. Following two more washes in PRF H-TCM with or without dbcAMP, the calcein dye confined within the denuded oocyte was measured.

Within 5-10 min of denuding, the intra-oocyte fluorescence emission of calcein in pulsed oocytes was measured using a fluorophotometric-inverted microscope (Leica, Wetzlar, Germany). Denuded oocytes in the field of view were analysed singularly and independently from neighbouring oocytes. Where applicable, fluorescence readings of denuded oocytes in each replicate experiment were adjusted to relative fluorescence intensity compared to the background.

2.11 Statistical analysis

Each experiment was replicated 3-5 times. Maturation, developmental data and follicle proportion data was subjected to three-way or one-way analysis of variance (ANOVA) following arcsine transformation and, where appropriate, proportional differences between groups were tested using the Tukey comparison method or students *t-test*. Ovary, follicle and oocyte measurement raw data were subjected to one-way ANOVA followed by the Holm-Sidak method *post hoc* comparison. Cyclic AMP measurements, steroid analysis, and cell numbers were tested with one-way ANOVA, and differences between means were detected using *t* tests. A rounded P value of less than 0.05 was considered to indicate a statistically significant difference between groups. Statistical analysis was carried out using Sigma Stat 3.1 (Systat Software Inc., Richmond, CA, USA). Graphs and figures were constructed using Sigma Plot 9.0 (Systat Software Inc., Richmond, CA, USA) and depict statistical differences calculated as described here.

Chapter 3

Effect of the onset of puberty on ovarian morphology and oocyte developmental competence

3 Effect of the onset of puberty on ovarian morphology and oocyte developmental competence

3.1 Introduction

Oocytes from the ovaries of slaughtered domestic animals are a relatively cheap, convenient and abundant source of material for *in vitro* embryo production (IVP). The use of pre-pubertal animals as donors for IVP makes it possible to shorten the generation interval, thereby increasing the rate of genetic gain achievable through conventional reproductive or embryo manipulation technologies (Georges and Massey 1991; Lohuis 1995). Offspring have been produced following *in vitro* maturation (IVM) and fertilisation of oocytes from pre-pubertal cows (Armstrong et al. 1992; Armstrong et al. 1994a), sheep (Armstrong et al. 1994b; O'Brien et al. 1996), goats (Mogas et al. 1995) and pigs (Yoshida *et al.* 1990; Yoshida *et al.* 1992). In pigs, *in vitro* matured pre-pubertal oocytes have been used to produce nuclear transfer embryos that have developed to term following transfer to recipient pigs (Lai et al. 2002; Park et al. 2002).

It is well established in domestic livestock species that oocyte developmental competence increases from birth with the oocytes of pre-pubertal donors displaying reduced blastocyst development compared to those of adult donors (Armstrong 2001). This is also true for the pig and since the meat industry has a general preference for pre-pubertal pigs, these are the most abundant and commonly utilised oocyte source for pig embryo IVP (O'Brien *et al.* 2000; Marchal *et al.* 2001; Grupen *et al.* 2003; Ikeda and Takahashi 2003; Sherrer *et al.* 2004). Pre-pubertal oocytes also tend to have different metabolism, protein expression pattern and stability of key maternally inherited transcripts when compared to adult oocytes (Gandolfi et al. 1998). Moreover, *in vivo* derived oocytes from pigs in first oestrus are less developmentally competent than those

at their third oestrus (Archibong et al. 1987; Menino et al. 1989). These findings indicate that oocyte developmental competence increases after the onset of puberty, but whether such an increase occurs immediately or requires multiple oestrous cycles has not yet been determined.

The developmental potential of oocytes matured *in vitro* correlates positively with oocyte size (Liu *et al.* 2002; Lucas *et al.* 2002; Griffin *et al.* 2006) and follicle diameter in a number of species including the cow and pig (Marchal *et al.* 2002; Lucas *et al.* 2003; Kauffold *et al.* 2005; Lequarre *et al.* 2005). Oocyte penetrability and the rate of blastocyst formation following IVF also increases with follicle size in a positive and step-wise manner in the pig (Marchal *et al.* 2002; Lucas *et al.* 2003). Meiotic competence of pig oocytes is also acquired progressively with follicular growth, with more oocytes from 3-8 mm follicles reaching MII than those from follicles smaller than 3 mm (Motlik and Fulka 1986; Yoon *et al.* 2000; Sun *et al.* 2001; Liu *et al.* 2002; Lucas *et al.* 2002; Marchal *et al.* 2002; Ikeda and Takahashi 2003). However, to date, the ovarian morphology and oocyte developmental competence at the onset of puberty in the pig has not been investigated.

The aim of this study was to determine the ovarian, follicular and oocyte morphology and oocyte developmental competence at the onset of puberty in the pig. Ovaries of peri-pubertal pigs were separated into two groups on the basis of presence of corpora lutea (CL) or absence of corpora lutea (NCL), representing ovulating and non-ovulating pigs respectively.

3.2 Experimental design

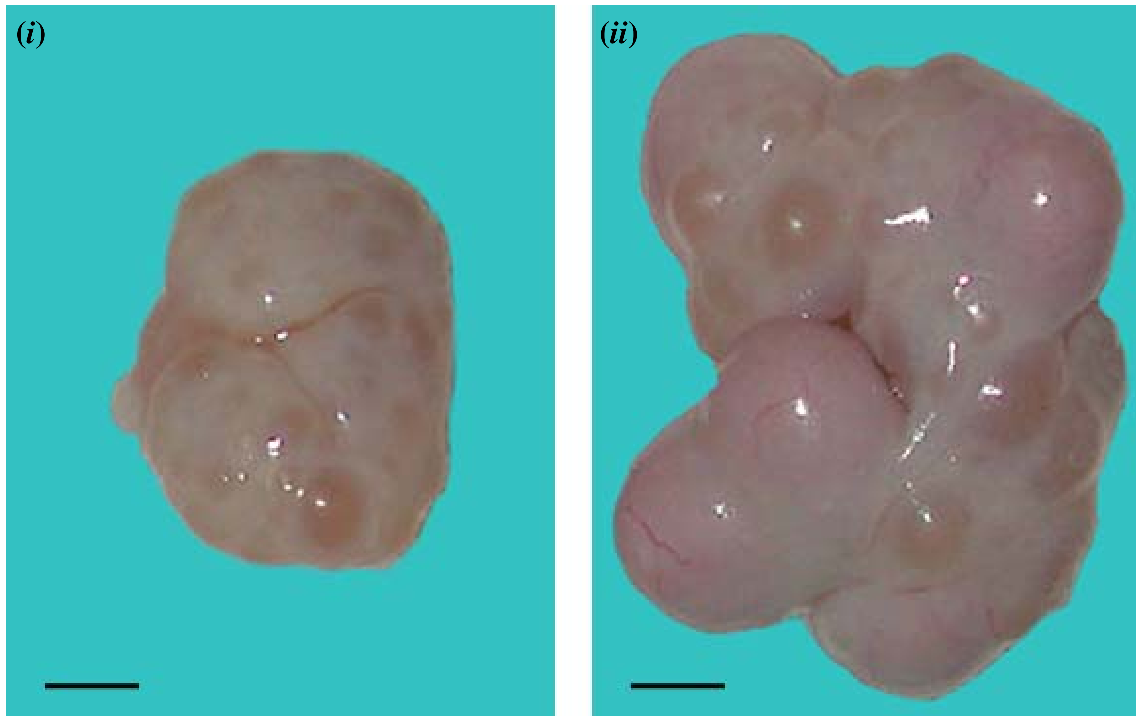
3.2.1 Experiment 1: Ovarian and oocyte morphology at the onset of puberty.

Ovarian dimensions, follicle number, and follicle diameter were measured for NCL and CL ovaries. For photos of typical NCL and CL ovaries employed in this study refer to Figure 3.1. The length and width of ovaries from the NCL (n = 51) and CL (n = 51) groups were measured over 3 replicates. All antral follicles and 3-8 mm follicles (typical aspiration range) were counted on the surface of NCL (n = 29 ovaries and 678 follicles) and CL (n= 25 ovaries and 456 follicles) ovaries over 3 replicates. Following 48 h IVM, the diameters of randomly selected intact oocytes from NCL (n = 54) and CL (n = 50) groups of ovaries were measured over 3 replicates. Oocyte measurements were taken both including and excluding the zonae pellucida, using a stereomicroscope with graticules corresponding to micrometers (μm) at 6.3X magnification.

3.2.2 Experiment 2: Oocyte developmental competence at the onset of puberty.

NCL and CL ovaries were matured in control conditions for 48 h IVM and were then artificially activated. Four replicates of NCL (n = 489) and CL (n = 242) oocytes were assessed for parthenote embryo development.

Figure 3.1 Images of typical NCL (*i*) and CL (*ii*) ovaries. The bar represents 5 mm.



3.3 Results

3.3.1 Ovarian and oocyte morphology at the onset of puberty

The ovarian dimensions, follicle number and diameter and oocyte diameter were measured for NCL and CL ovaries and are presented in Table 3.1. CL ovaries were significantly larger than NCL ovaries, in both width ($P < 0.001$) and length ($P < 0.001$). Follicles of CL ovaries were significantly larger in diameter than those of NCL ovaries ($P = 0.006$). The average number of antral follicles per ovary was greater on the surface of NCL ovaries than on CL ovaries ($P = 0.007$). However, the number of follicles in the 3-8 mm aspiration range did not differ between the two ovary types ($P > 0.05$). The average diameter of oocytes from CL ovaries was larger than that of oocytes from NCL ovaries ($P = 0.015$). Upon removal of the zonae pellucida, there was a trend ($P = 0.059$) towards CL oocytes having a larger diameter than NCL oocytes.

3.3.2 Oocyte developmental competence at the onset of puberty

To determine oocyte developmental competence at the onset of puberty the *in vitro* development of oocytes from NCL and CL ovaries was assessed following IVM and parthenogenetic activation. There was no significant difference in the rate of nuclear maturation between oocytes from NCL ovaries and those from CL ovaries (P > 0.05). The development of parthenogenetically activated oocytes from NCL and CL ovaries after 7 days IVC is shown in Table 3.2. There were no significant differences in the proportions of activated oocytes that cleaved or developed to the 4- to 8- cell, morula and blastocyst stages between the NCL and CL groups (P > 0.05).

Table 3.1 Morphological measurements of ovaries, follicles and oocytes from NCL and pubertal CL ovaries.

Structure	Measurement	NCL	CL
Ovary	Width (mm)	16 ± 0 [a]	22 ± 1 [b]
	Length (mm)	24 ± 0 [a]	33 ± 1 [b]
Follicle	Total n per ovary	47 ± 2 [a]	21 ± 2 [b]
	3-8 mm follicles per ovary	24 ± 1 [a]	18 ± 2 [a]
	Diameter (mm)	3 ± 0 [a]	5 ± 0 [b]
Oocyte	Diameter (µm)	146 ± 2 [a]	159 ± 1 [b]
	Diameter, zona-free (µm)	113 ± 2 [a]	125 ± 2 [a]

[abc]Across rows, values with different superscripts indicate significant differences (P < 0.05).
[1]Values expressed as mean ± S.E.M.
[2]Data are expressed as mean percentage of cleaved.
[3]Data are expressed as mean percentage of total.

Table 3.2 Comparison of developmental competence between NCL and CL oocytes following parthenogenetic activation.

	NCL	CL
% MII [1]	92 ± 3 [a]	92 ± 3 [a]
% Cleaved [1,2]	91 ± 3 [a]	88 ± 11[a]
% < 8 Cell [1,2]	35 ± 7 [a]	43 ± 7 [a]
% Morula [1,2]	15 ± 3 [a]	12 ± 5 [a]
% Blastocyst [1,2]	26 ± 6 [a]	23 ± 2 [a]
% Blastocyst[1,3]	24 ± 6 [a]	21 ± 4 [a]

[abc]Across rows, values with different superscripts indicate significant differences ($P < 0.05$).

[1]Values expressed as mean ± S.E.M.

[2]Data are expressed as mean percentage of cleaved.

[3]Data are expressed as mean percentage of total.

3.4 Discussion

The experiments presented in this chapter have identified that ovaries obtained from a group of uniform size pigs slaughtered around puberty have two distinctly different morphologies. About half of the ovaries were typically pre-pubertal in appearance in that they did not display any corpora albicans, corpora lutea or other signs of ovulation. The other half displayed corpora lutea, indicating that these animals had undergone at least one oestrous cycle. These results demonstrate that there is an immediate increase in the mean diameter of follicles (in the 3-8 mm aspiration range) at the onset of puberty. Furthermore, the mean diameter of oocytes recovered from 3-8 mm follicles also increases with the onset of puberty. These findings are in agreement with those of Lucas *et al.* (2002) who reported a positive correlation between follicle and immature oocyte diameter in pre-pubertal pigs (Lucas et al. 2002). The current study extends these findings to include mature oocytes from 3-8 mm follicles at the onset of puberty.

A clear relationship has been demonstrated between oocyte diameter and developmental competence in both adult (Hytell et al. 1997; Otoi et al. 1997) and pre-pubertal cattle (Gandolfi et al. 1998). The influence of follicle size on oocyte developmental potential during IVM and IVF has also been established in cattle (Pavlok *et al.* 1992; Lonergan *et al.* 1994). In the pig, stage of nuclear maturation of the oocyte increases as oocyte diameters increase, and oocyte diameter increases with follicles size, remaining constant within the 2.8-6.5 mm follicle size range (Lucas et al. 2002). Smaller ooplasm diameter in pre-pubertal pig oocytes has also been attributed to reduced efficiency of embryo production following IVP (Ikeda and Takahashi 2003). In the present study, despite the differences in follicle and oocyte diameter between NCL and CL ovaries, the meiotic and developmental competence of the two groups of oocytes was not significantly different.

An important factor that influences oocyte developmental competence is oocyte donor age (reviewed by Armstrong 2001). In cattle, the acquisition of oocyte developmental competence increases with pre-pubertal donor age, and full developmental competence of the oocyte is reached before puberty (Presicce et al. 1997). However, in the pig, oocytes of all pre-pubertal donors display lower developmental competence than those of adult donors (O'Brien *et al.* 2000; Marchal *et al.* 2001). Our results demonstrate that although there were significant differences in follicle and oocyte diameter between NCL and CL ovaries, the developmental competence of the oocytes was not significantly different. These findings suggest that pig ovaries need to be exposed to more than one oestrous cycle in order for oocytes to attain an increased developmental competence. This interpretation is supported by findings of *in vivo* studies. Gilts mated at pubertal oestrus have a higher incidence of embryonic mortality when compared with gilts mated at third oestrus (Archibong et al. 1987; Menino et al. 1989), which cannot be attributed

to an abnormal uterine environment (Archibong et al. 1992). In addition, pubertal pigs appear to ovulate a greater proportion of immature and aneuploid oocytes than third oestrous pigs, further suggesting that the deficiency relates to oocyte quality (Koenig and Stormshak 1993). In contrast, a recent *in vivo* study has reported that delaying first mating until the second oestrous does not significantly increase either ovulation or embryo production rate in the pig (van Wettere *et al.* 2005).

Alternative explanations for the results of the present study are also possible. Recently, pre-pubertal FF has been found to exert a negative effect on the developmental competence of adult oocytes, which developed to the blastocyst stage at a greater rate when matured in adult FF than in pre-pubertal FF (Grupen et al. 2003). Therefore, the supplementation of maturation medium in the present study with pre-pubertal FF may not have allowed differences in developmental competence between the two groups of oocytes to be observed. One study has reported that electrical activation and embryo culture of pig oocytes following IVM for 42 h resulted in higher development to day 7 blastocysts than those matured for 48 h (36 % vs. 15 % respectively) (Ikeda and Takahashi 2001). Therefore, parthenogenetic activation of oocytes following 48 h IVM may not have allowed visualisation of differences in developmental competence between NCL and CL groups of oocytes.

Parthenogenetic activation was employed in this study to circumvent the detrimental effect of polyspermic fertilisation during IVF, which occurs at a high rate in pre-pubertal pig oocytes, and to reduce the variability of embryo production associated with IVF (O'Brien *et al.* 2000; Marchal *et al.* 2001). Furthermore, parthenogenesis allows visualisation of the first stages of embryo development without the variables introduced during IVF by the paternal genome. In this study, chemical parthenogenetic activation was achieved using a combined treatment of ionomycin, a powerful calcium ionophore,

and 6-DMAP, a protein kinase inhibitor. This combined treatment has been used successfully to demonstrate differences in developmental competence between pre-pubertal and adult oocytes in the pig (Ikeda and Takahashi 2001). However, further studies should also be carried out using IVF to support these results.

In conclusion, this chapter demonstrates that while ovarian, follicular and oocyte morphology are immediately altered at the onset of puberty, oocyte developmental competence is not affected at the same time. These results suggest that the oocytes of pubertal pigs must be exposed to more than one oestrous cycle to attain their full developmental competence.

Chapter 4

Effect of dibutyryl cAMP on *in vitro* matured pre-pubertal and adult oocytes

4 Effect of dibutyryl cAMP on *in vitro* matured pre-pubertal and adult oocytes

4.1 Introduction

Oocytes derived from pre-pubertal pigs possess lower developmental competence than those from adult donors (O'Brien *et al.* 2000; Marchal *et al.* 2001; Grupen *et al.* 2003; Ikeda and Takahashi 2003; Sherrer *et al.* 2004). The results of Chapter 1 confirm that oocyte developmental competence is not conferred exclusively with the onset of puberty in the pig. The low blastocyst development of pre-pubertal pig oocytes has been associated with a high incidence of polyspermy following *in vitro* fertilisation (IVF) (O'Brien *et al.* 2000; Marchal *et al.* 2001), which is though to be due to incomplete cytoplasmic maturation and abnormal fertilisation (Niwa 1993; Nagai 1994).

At the time of aspiration, pre-pubertal porcine oocytes display heterogeneity at the GV stage of meiosis (McGaughey and Polge 1972; Motlik and Fulka 1976). Continued asynchronous progression results in cohorts of oocytes reaching MII and aging for the remainder of *in vitro* maturation (IVM) (Grupen *et al.* 1997; Nagai *et al.* 1997; Funahashi *et al.* 1997b). The majority of studies that have directly compared pre-pubertal and adult oocytes report that they reach MII at a similar rate by the end of IVM (Marchal *et al.* 2001; Grupen *et al.* 2003; Ikeda and Takahashi 2003; Sherrer *et al.* 2004). However, a study by O'Brien *et al.* (2000) reported a lower proportion of pre-pubertal oocytes reach MII by the end of IVM.

The principal regulator of the oocyte meiotic cell cycle is cAMP. *In vivo*, gonadotropins trigger a transient increase in oocyte cAMP content, which subsequently decreases to trigger GVBD (Mattioli *et al.* 1994). In contrast, physical removal of oocytes from their follicular environment induces spontaneous resumption of meiosis (Pincus and Enzmann 1935). It is thought that premature reduction of oocyte cAMP content during

IVM has an adverse effect on the coordination of nuclear and cytoplasmic maturation, resulting in decreased developmental potential.

Cyclic AMP is synthesised in cumulus cells by adenylate cyclase activity in response to gonadotropins and enters the oocyte via gap junctions (Schultz *et al.* 1983a; Racowsky 1985). High intracellular levels of cAMP maintain oocyte meiotic arrest at the GV stage by activating cAMP-dependent protein kinase A (PKA), which in turn maintains MPF, another key regulator of the meiotic cell cycle, in the inactive form (pre-MPF) (Bornslaeger *et al.* 1986; Spaulding 1993; Francis and Corbin 1994). A number of treatments have been used to elevate oocyte cAMP content, including phosphodiesterase (PDE) inhibitors, stimulators of adenylate cyclase activity and analogues of cAMP. Previously, treatment of pre-pubertal porcine oocytes for 20-22 h with dbcAMP, a membrane permeable analogue of cAMP, has been shown to increase the synchrony of meiotic progression and the incidence of blastocyst formation following IVF (Funahashi *et al.* 1997b; Somfai *et al.* 2003). Exposure of pre-pubertal porcine oocytes to dbcAMP for the first 22 h of IVM has been found to increase the incidence of monospermic fertilisation in porcine oocytes (Mattioli *et al.* 1994). The effect of dbcAMP during IVM on maturation and development of adult oocytes has not yet been reported.

Expansion of the compact layers of cumulus cells surrounding the oocyte is another process that occurs in response to gonadotropin stimulation and accompanies meiotic progression. *In vitro*, cumulus expansion can also be induced by chemicals that increase intracellular cAMP such as FSH (Dekel and Kraicer 1978) or activators of adenylate cyclase such as forskolin (Racowsky 1985). In a comparison of cumulus expansion between granulosa enclosed and non-enclosed pre-pubertal cumulus-oocyte complexes (Gandolfi *et al.*), poor cumulus expansion during IVM affected both nuclear and

cytoplasmic maturation of pre-pubertal pig oocytes and subsequent IVF results (Somfai et al. 2004). Comparison of cumulus expansion between pre-pubertal and adult COCs during IVM and the effect of dbcAMP on this expansion has not yet been characterised.

Progesterone is synthesised by the pig COC in response to gonadotropin stimulation and is secreted into either the FF *in vivo* or the culture media *in vitro* (Ainsworth et al. 1980; Driancourt et al. 1998; Dode and Graves 2002; Shimada and Terada 2002b). Increasing concentrations of progesterone have been associated with meiotic resumption and progression of oocyte maturation and cumulus expansion (Dode and Graves 2002). Addition of progesterone to oocyte IVM culture has been shown to have a positive effect on IVF outcomes in the pig (Karlach 1987; Zhang and Armstrong 1989; Mattioli *et al.* 1998). Although a different study in the pig found that progesterone addition during oocyte IVM had no effect on subsequent fertilisation and embryo cleavage, suggesting the level of secretion by the COC may be adequate for successful oocyte maturation (Dode and Graves 2002). Comparison of progesterone secretion by pre-pubertal and adult COCs during IVM and the effect of dbcAMP on this secretion has not been characterised.

The aim of the experiments presented in this chapter was to examine the response of pre-pubertal and adult oocytes to dbcAMP exposure for the first 22 h of IVM. The cAMP content, meiotic progression, cumulus expansion and progesterone secretion of oocytes from pre-pubertal and adult donors were determined at various time points during IVM in the presence or absence of dbcAMP treatment. The developmental competence of dbcAMP treated and control oocytes was also determined following parthenogenetic activation and IVF.

4.2 Experimental design

4.2.1 Experiment 1: Cyclic AMP content during IVM and subsequent parthenote embryo development of pre-pubertal and adult oocytes

COCs from each donor group were matured in control conditions or with 1 mM dbcAMP for the first 22 h IVM. A proportion of oocytes from each group was removed at 0 h and 22 h IVM for cAMP measurement and the remaining oocytes were artificially activated after 46 h IVM. Three replicates each of pre-pubertal and adult oocytes were assessed for cAMP content (n = 305 and 276 respectively) and parthenote development (n = 335 and 239 respectively).

4.2.2 Experiment 2: Meiotic progression of pre-pubertal and adult oocytes during IVM

COCs from each donor age group were matured in control conditions or with dbcAMP for the first 22 h IVM. Eight replicates of pre-pubertal oocytes (n = 960) and 6 replicates of adult oocytes (n = 520) were assessed at 0, 16, 22, 40 and 44 h IVM.

4.2.3 Experiment 3: Cumulus expansion of pre-pubertal and adult COCs during IVM

COCs from each donor group were matured for 46 h in control conditions or with dbcAMP during the first 22 h. Four replicates of pre-pubertal oocytes (n = 656) and 3 replicates of adult oocytes (n = 718) were assessed at 22 h and 46 h IVM

4.2.4 Experiment 4: Progesterone secretion by pre-pubertal and adult COCs during IVM

COCs from each donor group were matured for 22 h either in control conditions or with dbcAMP and then changed to control media, minus dbcAMP, for a further 24 h IVM. Spent media was collected at both time points from both donor groups and different treatments. Spent IVM media was collected after 22 h and the final 24 h of IVM from five replicates of pre-pubertal COCs (n = 909) and adult COCs (n = 825).

4.2.5 Experiment 5: Embryo development of pre-pubertal and adult oocytes following IVF

Pre-pubertal and adult COCs were matured in control conditions or with dbcAMP for the first 22 h and then fertilised after 46 h IVM. Three replicates of pre-pubertal (n = 299) and adult (n = 325) IVM oocytes were assessed for blastocyst development following IVF. When possible, pre-pubertal and adult IVF experiments were conducted on the same or consecutive days using the same sperm to reduce variation in fertilisability.

4.3 Results

4.3.1 Cyclic AMP content during IVM and subsequent parthenote embryo development of pre-pubertal and adult oocytes.

The cAMP content of pre-pubertal and adult oocytes at 0 h and 22 h is shown in Figure 4.1. Zona-free oocytes isolated from pre-pubertal and adult COCs contained similar levels of cAMP at 0 h. Following a 22 h incubation as an intact COC in the absence of dbcAMP, the cAMP content of adult oocytes increased 2.5-fold over that at 0 h, whereas no increase occurred in cAMP content of pre-pubertal oocytes. Culture for 22

h in the presence of dbcAMP resulted in approximately two-fold greater cAMP content in both pre-pubertal and adult oocytes than those observed with culture in the absence of dbcAMP. By completion of IVM after 44 h culture, cAMP levels had decreased to barely detectable levels for all four groups (data not shown, yet Appendix 2 depicts basal cAMP levels at 46 h).

The effect of donor age and dbcAMP on the developmental competence of IVM oocytes following parthenogenetic activation is presented in Table 4.1. Pre-pubertal and adult oocytes underwent similar high rates of maturation *in vitro* and cleavage after activation. However, significantly more pre-pubertal than adult parthenotes blocked at <8-cell stage (57 ± 12 % vs. 21 ± 7 % respectively) and this was not altered by culture with dbcAMP. Treatment with dbcAMP increased the rate of blastocyst formation from pre-pubertal oocytes compared with the control group cultured without dbcAMP (35 ± 6 % vs. 25 ± 5 % respectively), whereas rate of blastocyst formation from adult oocytes was not affected by culture with dbcAMP. The blastocyst rate of adult oocytes was higher than that of pre-pubertal oocytes following IVM both in the absence (59 ± 2 % vs. 25 ± 5 % respectively) and presence (57 % ± 10 vs. 35 ± 6 %, respectively) of dbcAMP.

Figure 4.1. Cyclic AMP (cAMP) concentrations of pre-pubertal and adult oocytes before and after 22 h IVM in control conditions or dbcAMP treatment. Different superscripts indicate significant differences (P < 0.05).

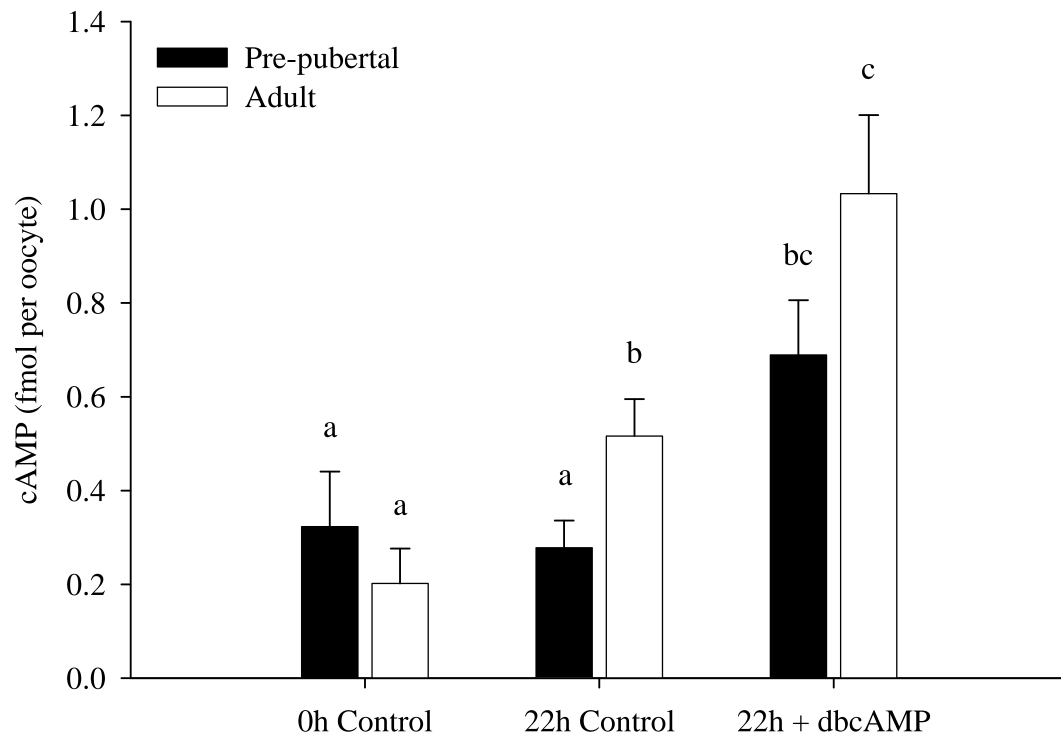


Table 4.1 The effect dbcAMP on the developmental competence of pre-pubertal and adult oocytes following IVM and parthenogenetic activation.

Groups	n	% MII[1,2]	% Cleaved[1,2]	% <8 Cell[1,2]	% Morula[1,2]	% Blastocyst[1,2]	% Blastocyst[1,3]
Pre-pubertal Control	188	91 ± 3 ^{a}	87 ± 5 ^{a}	57 ± 12 ^{a}	15 ± 7 ^{a}	25 ± 5 ^{a}	21.6 ± 5.2^{a}
Pre-pubertal + dbcAMP	125	89 ± 3 ^{a}	76 ± 11 ^{a}	53 ± 10 ^{ab}	8 ± 4 ^{a}	35 ± 6 ^{b}	26.3 ± 6.4^{a}
Adult Control	185	93 ± 3 ^{a}	80 ± 6 ^{a}	21 ± 7 ^{b}	16 ± 6 ^{ab}	59 ± 2 ^{c}	47.3 ± 5.1^{b}
Adult + dbcAMP	136	90 ± 2 ^{a}	79 ± 5 ^{a}	28 ± 5 ^{ba}	12 ± 8 ^{b}	57 ± 11 ^{c}	45.7 ± 11.0^{ab}

^{abc}Within columns, values with different superscripts indicate significant differences ($P < 0.05$).

[1]Values expressed as mean ± S.E.M.

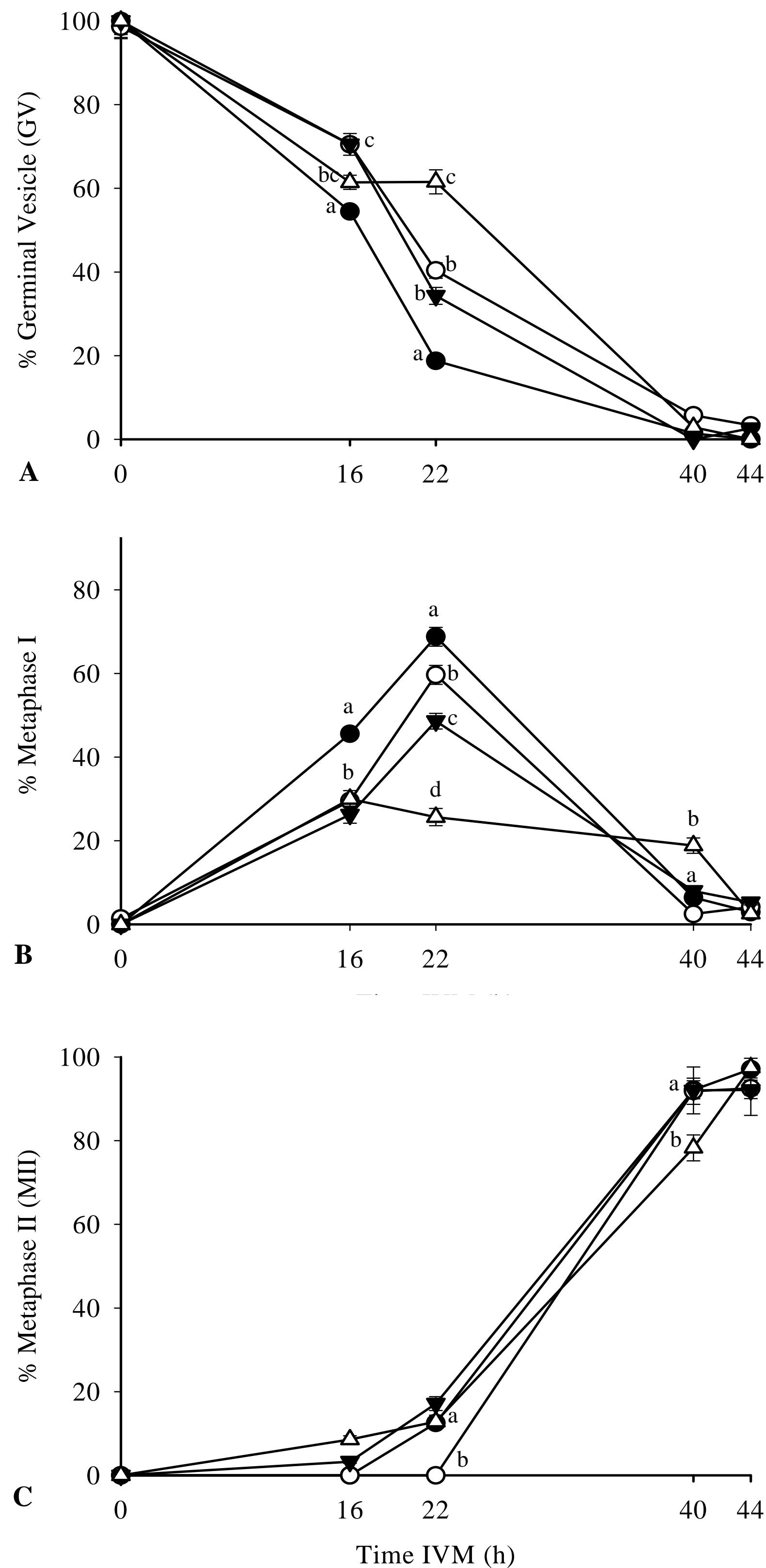
[2]Data are expressed as mean percentage of cleaved.

[3]Data are expressed as mean percentage of total.

4.3.2 Meiotic progression of pre-pubertal and adult oocytes during IVM.

The progression of meiotic maturation in pre-pubertal and adult oocytes matured for 0, 16, 22, 40 and 44 h IVM is presented in Figure 4.2. About half of the pre-pubertal and adult oocytes were in stages of GVBD (GVII-IV) at the start of IVM, while the remainder were at the intact GVI stage (data not shown). At 16 h and 22 h IVM in the absence of dbcAMP, a greater proportion of pre-pubertal oocytes had progressed to the MI stage of meiosis compared with adult oocytes (46 ± 1 vs. 26 ± 2 % and 69 ± 2 vs. 49 ± 2 %, respectively). However, by the end of IVM there was no difference in the percentages of pre-pubertal and adult oocytes reaching MII at the time points examined. After 16 h IVM in the presence of dbcAMP, a similar number of pre-pubertal and adult oocytes had reached MI. Treatment with dbcAMP significantly reduced the proportion of pre-pubertal oocytes in MI at 16 h compared with the control group (46 ± 1 % vs. 30 ± 1 %). After 22 h the percentage of oocytes at MI was lower in the dbcAMP group compared with the control group for both pre-pubertal (69 ± 2 % vs. 60 ± 2 %) and adult (49 ± 2 % vs. 26 ± 2 %) oocytes. In the presence of dbcAMP, a higher proportion of pre-pubertal oocytes reached MI at 22 h compared with adult oocytes. After 40 h IVM with dbcAMP, the percentage of adult oocytes at MII was lower than when matured in the absence of dbcAMP (78 ± 3 % vs. 92 ± 3 %). At the end of IVM in the presence of dbcAMP, pre-pubertal and adult oocytes reached MII at similar rates.

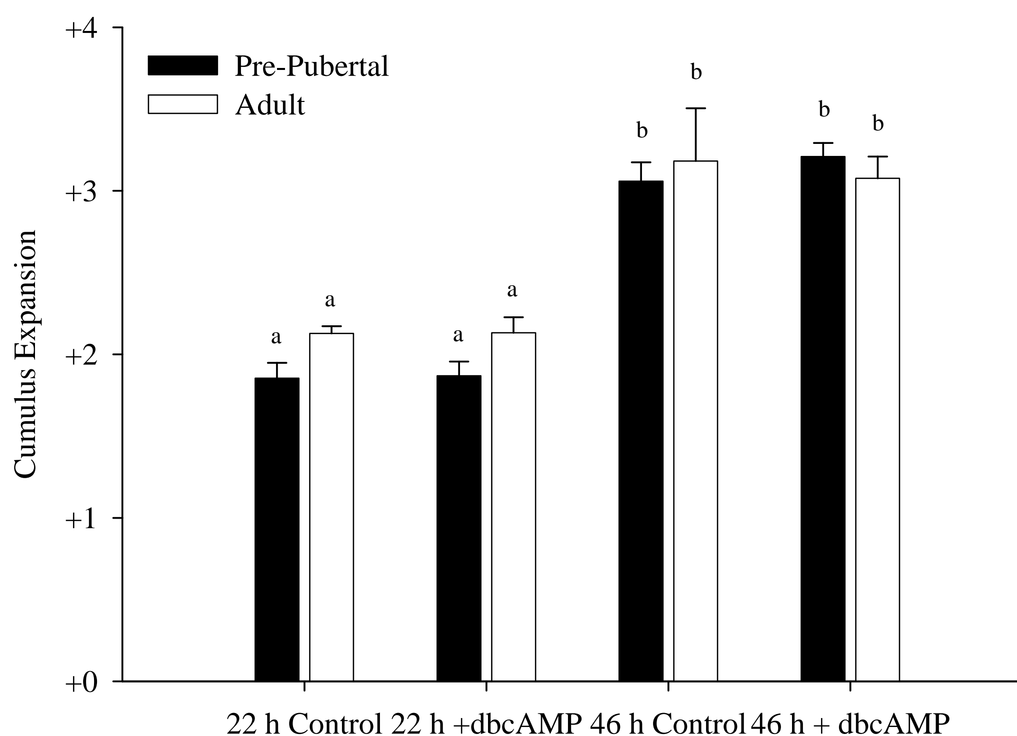
Figure 4.2 Progression of pre-pubertal (━●━) and adult (━▼━) oocytes from GV (A) to MI (B) and MII (C) stages of meiotic maturation at 0 h, 16 h, 22 h, 40 h and 44 h IVM. Open symbols indicate dbcAMP treatment (pre-pubertal ━○━, adult ━△━). Different superscripts indicate significant differences in meiotic progression at a particular time point ($P<0.05$).



4.3.3 Cumulus expansion of pre-pubertal and adult COCs during IVM.

The cumulus expansion of pre-pubertal and adult COCs during IVM is presented in Figure 4.3. After 22 h control IVM, pre-pubertal and adult COCs displayed similar cumulus expansion (2 ± 0 and 2 ± 0 respectively). After 46 h control IVM, there was a significant increase from 22 h in the degree of cumulus expansion of both pre-pubertal and adult COCs (3 ± 0 and 3 ± 0 respectively). Cumulus expansion scores in the dbcAMP treatment groups were similar to controls at 22 h and 46 h IVM.

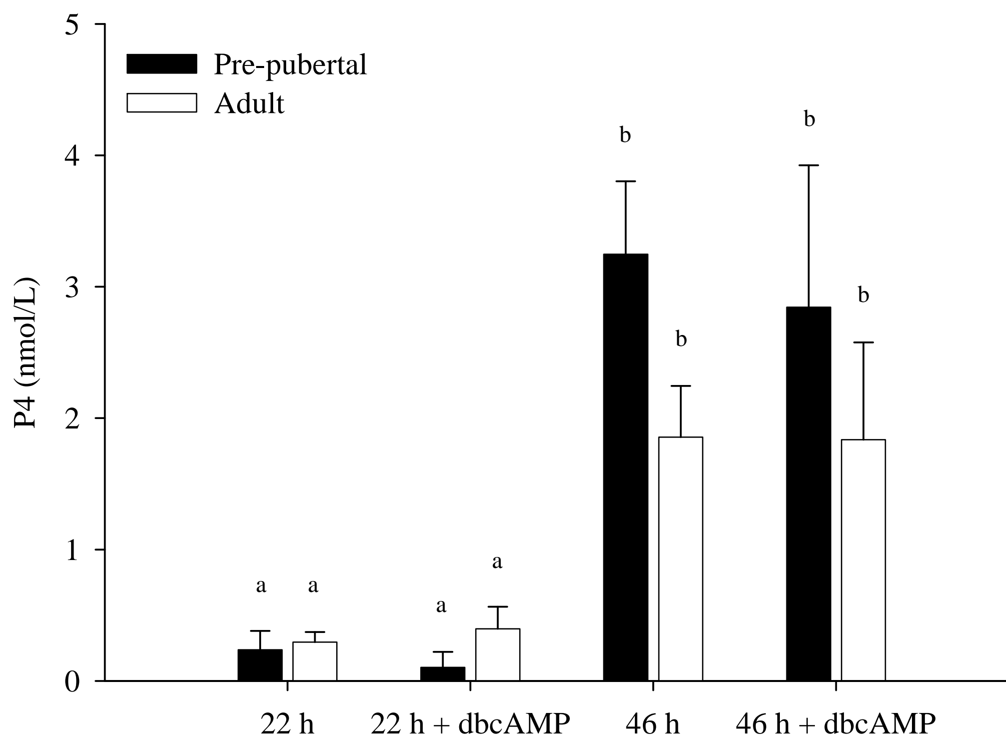
Figure 4.3. Cumulus expansion of pre-pubertal and adult COCs at 22 h and 46 h IVM in control or dbcAMP treatment conditions. Different superscripts indicate significant differences (P < 0.05).



4.3.4 Progesterone secretion by pre-pubertal and adult COCs during IVM.

The progesterone secretion of pre-pubertal and adult COCs during IVM is presented in Figure 4.4. After 22 h control IVM, pre-pubertal and adult COCs secreted similar amounts of progesterone (0.2 ± 0.1 nmol/L and 0.3 ± 0.1 nmol/L respectively). After 46 h control IVM, there was a significant increase from 22 h in the amount of progesterone secretion by both pre-pubertal and adult oocytes (3.3 ± 0.6 nmol/L and 1.9 ± 0.4 nmol/L respectively). progesterone secretion by COCs treated with dbcAMP was similar to controls at 22 h and 46 h IVM.

Figure 4.4 Progesterone (P4) concentrations in spent media of pre-pubertal and adult COCs after 0 to 22h and the final 22h to 46 h IVM and the effect of dbcAMP treatment for the first 22h IVM. Different superscripts indicate significant differences ($P < 0.05$).



4.3.5 Embryo development of pre-pubertal and adult oocytes following IVF.

The effect of donor age and dbcAMP on the developmental competence of IVM oocytes following IVF is presented in Table 4.2. In the control group, adult oocytes had a higher blastocyst formation rate compared with pre-pubertal oocytes (47 ± 7 % vs. 19 ± 5 %). Pre-pubertal oocytes displayed a significantly higher percentage of <8 cell embryos compared with adult oocytes (42 ± 8 % vs. 32 ± 0 %). The addition of dbcAMP for the first 22 h IVM significantly increased the blastocyst formation rate of pre-pubertal oocytes (19 ± 5 % vs. 36 ± 10 %), but not adult oocytes.

Table 4.2 The effect dbcAMP on the developmental competence of pre-pubertal and adult oocytes following IVM/IVF.

Groups	n	% MII[1]	% Cleaved[1,2]	% <8 Cell[1,2]	% Morula[1,2]	% Blastocyst[1,2]	% Blastocyst[1,3]
Pre-pubertal Control	159	93 ± 3 [a]	81 ± 6 [a]	42 ± 8 [a]	37 ± 7 [a]	19 ± 5 [a]	15 ± 3 [a]
Pre-pubertal + dbcAMP	162	91 ± 6 [a]	88 ± 7 [a]	39 ± 9 [a]	24 ± 7 [a]	36 ± 7 [bc]	30 ± 6 [ab]
Adult Control	188	96 ± 1 [a]	78 ± 3 [b]	32 ± 1 [b]	18 ± 6 [ab]	47 ± 7 [c]	36 ± 4 [b]
Adult + dbcAMP	150	96 ± 1 [a]	80 ± 2 [b]	34 ± 8 [ba]	12 ± 6 [b]	50 ± 6 [c]	40 ± 10 [b]

[abc]Within columns, values with different superscripts indicate significant differences ($P < 0.05$).

[1]Values expressed as mean ± S.E.M.

[2]Data are expressed as mean percentage of cleaved.

[3]Data are expressed as mean percentage of total.

4.4 Discussion

The results presented in this chapter are the first to report adult pig oocyte cAMP concentrations during IVM and compare these directly with pre-pubertal oocytes matured in the absence or presence of 1 mM dbcAMP for the first 22 h. The results of the first experiment demonstrated no change in cAMP content of pre-pubertal oocytes and a doubling in cAMP concentration of adult oocytes after 22 h. This indicates that pre-pubertal oocytes have a reduced ability to accumulate intra-oocyte cAMP compared with adult oocytes when stimulated with FSH under IVM conditions.

Time-dependent increases in the cAMP content have been reported for pre-pubertal pig oocytes matured either within COCs (Shimada and Terada 2002a) or within everted (whole) follicles (Mattioli et al. 1994). The findings for zona-free oocytes reported in this chapter are consistent with those of Mattioli et al. (1994), who observed no significant change from 0 h in the amount of cAMP in pre-pubertal oocytes matured as COCs or denuded oocytes throughout IVM (Mattioli et al. 1994). In contrast, Shimada et al (2002) have reported that the cAMP concentration of pre-pubertal oocytes significantly increases at 4 h IVM, reaches a peak level at 8 h, and then dramatically drops at 12 h and again at 28 h to reach basal levels at 32 h and remains low until the end of maturation (Shimada and Terada 2002a). The reason for these differences is unknown, but might be explained by variation in the methods used to assay cAMP, for example some used zona-free whilst others used zona-intact oocytes. See Appendix 2 for a comparison of cAMP measurements from zona-intact versus zona-free oocytes.

Treatment with dbcAMP for the first 22 h of IVM resulted in significant increases in the intra-oocyte cAMP concentration of both pre-pubertal and adult oocytes at 22 h. The dbcAMP derivative of cAMP mimics the action of endogenous cAMP (Budavari 1989).

In comparison with cAMP, dbcAMP has a lipophilic nature and is therefore used preferentially with intact cells due to its greater permeability and resistance to hydrolysis by PDEs (Henion et al. 1967; Swislocki 1970). To our knowledge, this is the first study to directly measure cAMP content of dbcAMP treated oocytes during IVM. The cross reactivity between dbcAMP and the cAMP antibody employed in this assay was around 1% (Appendix 3), suggesting that these results represent the actual cAMP content of the COC. In addition, the low values observed at 0 h for dbcAMP treated oocytes provides confidence that there is minimal residue cAMP being measured.

In terms of meiotic progression, the results presented here demonstrate that a higher proportion of pre-pubertal oocytes had undergone GVBD and progressed to the MI stage of meiosis compared with adult oocytes by 16 and 22 h of control IVM. Previous studies have described variation in meiotic stage in pre-pubertal pig oocytes at the start of IVM with continued asynchronous meiotic progression that resulted in a cohort of aged oocytes at the end of IVM (McGaughey and Polge 1972; Motlik and Fulka 1976; Ocampo et al. 1993; Grupen et al. 1997; Funahashi et al. 1997b). The findings presented in this chapter are in agreement with those of previous studies in pre-pubertal pig oocytes and extend these findings by directly comparing the meiotic progression of pre-pubertal and adult oocytes. Our results indicate that pre-pubertal oocytes have a reduced ability to accumulate cAMP compared with adult oocytes, which may account for their asynchronous meiotic progression.

Exposure of pre-pubertal oocytes to 1 mM dbcAMP for 22 h IVM significantly delayed meiotic progression and increased the rate of blastocyst formation. The meiotic progression of adult oocytes was also delayed by dbcAMP treatment, but this did not result in an increase in the rate of blastocyst formation. Despite exposure to dbcAMP, a higher proportion of pre-pubertal compared with adult oocytes had attained MI at 22 h,

indicating that dbcAMP treatment of pre-pubertal oocytes could not prevent GVBD as effectively as in adult oocytes. The results presented in this chapter are in agreement with previous studies in pre-pubertal pig oocytes, which demonstrated that treatment with 1mM dbcAMP decreased GVBD by around 50% (Funahashi et al. 1997b; Somfai et al. 2003). The present findings, although reporting the same trend, only demonstrated a 20% reduction in GVBD following dbcAMP treatment for 22 h. The reasons for this difference are unknown, but may be due to differences in IVM culture conditions between these studies.

In experiment 3 there was a significant increase in cumulus expansion of pre-pubertal and adult COCs from 22 to 46 h of control IVM, yet expansion of pre-pubertal and adult COCs was similar at both time points, whether matured under dbcAMP or control conditions. Prior to initiation of meiosis, cumulus cells maintain meiotic arrest via elevation of intercellular cAMP level through gap junctions (Dekel and Beers 1980; Racowsky 1984; Downs 2001; Shimada and Terada 2002b). Cumulus expansion and occlusion of gap junctions appear to interrupt the gap junction inhibitory signal and are responsible for progression of meiosis (Isobe et al. 1998; Isobe and Terada 2001). Cumulus expansion of IVM pre-pubertal and adult pig COCs has been reported, but this study is the first to directly compare the two and examine the effect of dbcAMP (Racowsky 1985; Somfai *et al.* 2004; Schoevers *et al.* 2005). Donor age and dbcAMP treatment had no effect on cumulus expansion in this study, which could reflect the qualitative nature of the assessment or support previous findings that cumulus expansion and gap junction communication are independent processes in the pig (Isobe et al. 1998).

In experiment 4 a significant increase in progesterone secretion of pre-pubertal and adult COCs was observed during the final 24 h of control IVM compared with the initial

22 h. progesterone secretion was similar at both time points for pre-pubertal and adult COCs, whether matured under dbcAMP or control conditions. These findings agree with previous results that concentrations of progesterone increase during IVM along with meiotic resumption and progression and cumulus expansion (Dode and Graves 2002; Shimada and Terada 2002b). In this study, donor age and dbcAMP treatment had no effect on progesterone secretion, indicating that the low developmental competence of pre-pubertal compared with adult oocytes does not reflect differing abilities to produce progesterone *in vitro*.

In the pig, oocytes from pre-pubertal donors display lower developmental competence than those of adult donors (O'Brien et al. 2000; Marchal et al. 2001; Grupen et al. 2003; Ikeda and Takahashi 2003; Sherrer et al. 2004). The finding presented here is in agreement with these previous studies, and has demonstrated that treatment with 1 mM dbcAMP for the first 22 h IVM improves blastocyst formation following both parthenogenetic activation and IVF in pre-pubertal pig oocytes. The blastocyst formation rate of dbcAMP treated pre-pubertal oocytes remained lower than that of adult oocytes, despite the dbcAMP treatment raising cAMP levels in pre-pubertal oocytes similar to those in adult oocytes at 22 h. Similar differences were observed in the developmental competence of pre-pubertal and adult oocytes following parthenogenetic activation and IVF. The findings presented in this chapter indicate that parthenogenetic development is a useful means of assessing developmental potential to the blastocyst stage. Parthenogenetic activation is also convenient in that it removes the variation in fertilising ability between sperm doses.

The intra-oocyte cAMP concentration differences reported in this chapter correlate with the maturational and developmental differences observed between pre-pubertal and adult IVM oocytes. Although the oocyte can synthesise cAMP, the lack of LH receptors

on the oolemma means that it probably does not account for the gonadotropin stimulated cAMP increase (Mattioli et al. 1994). However, cAMP is also produced in cumulus cells by adenylate cyclase when stimulated by LH or FSH and is transported into oocytes via gap junctions that communicate between cumulus cell projections though the zonae pellucida and the oocyte cell membrane (Schultz *et al.* 1983a; Racowsky 1985). Therefore, it seems likely that pre-pubertal COCs possess cumulus cells that either have a lower level of LH/FSH expression, a different adenylate cyclase response or different PDE activities. There is also the possibility that pre-pubertal oocyte GJC may not be as well formed or as functional as in adult oocytes. Alternatively, the observed differences may be due to differences in the distribution of follicles in the 3-8 mm aspiration range on ovaries of pre-pubertal and adult donors. Previously, the ability of granulosa cells to stimulate intra-oocyte cAMP accumulation has been shown to progressively decrease with follicle size (Mattioli et al. 1994).

In conclusion, this chapter demonstrates that pre-pubertal pig oocytes accumulate less cAMP during IVM, undergo a more rapid meiotic progression and display a lower ability to develop to the blastocyst stage than *in vitro* matured adult pig oocytes. Treatment with dbcAMP for the first 22 h IVM increased the cAMP content of pre-pubertal oocytes, which correlated with slower meiotic progression during IVM and improved developmental competence. Even though the cAMP levels of pre-pubertal oocytes were increased to levels similar to those of adult oocytes, the developmental potential of pre-pubertal oocytes remained lower than that of adult oocytes, suggesting that factor (s) independent of cAMP are also deficient in pre-pubertal oocytes. Elucidation of these deficiencies and a greater understanding of the mechanisms involved in the accumulation of cAMP in oocytes will facilitate future improvements to porcine embryo IVP systems.

Chapter 5

Effect of follicle size on pre-pubertal and adult oocyte developmental competence

5 Effect of follicle size on pre-pubertal and adult oocyte developmental competence

5.1 Introduction

The oocytes of pre-pubertal domestic livestock species display reduced rates of blastocyst formation compared to those of adults (reviewed by Armstrong 2001). While this is true also for the pig, in most developed countries pigs are slaughtered prior to reaching puberty, making pre-pubertal pigs the most abundant source of oocytes for the development of *in vitro* maturation (IVM) and related reproductive technologies (O'Brien *et al.* 2000; Marchal *et al.* 2001; Grupen *et al.* 2003; Ikeda and Takahashi 2003; Sherrer *et al.* 2004). In addition to lower rates of blastocyst formation, pre-pubertal pig oocytes also display a higher incidence of polyspermy following *in vitro* fertilisation (IVF) compared with adult pig oocytes (O'Brien *et al.* 2000; Marchal *et al.* 2001), which suggests that the cytoplasmic maturation is inadequate in these oocytes (reviewed by Niwa 1993 and Nagai 1994).

Increased *in vitro* developmental competence of oocytes correlates with increased oocyte diameter (Liu *et al.* 2002; Lucas *et al.* 2002; Griffin *et al.* 2006) and follicle diameter in a number of species including the cow and pig (Marchal *et al.* 2002; Lucas *et al.* 2003; Kauffold *et al.* 2005; Lequarre *et al.* 2005). This appears to relate to the increased ability of oocytes from larger follicle sizes to complete meiotic and cytoplasmic maturation. In the pig, a greater proportion of oocytes from 3-8 mm follicles reach MII compared to those from follicles smaller than 3 mm (Motlik and Fulka 1986; Yoon *et al.* 2000; Sun *et al.* 2001; Liu *et al.* 2002; Lucas *et al.* 2002; Marchal *et al.* 2002; Ikeda and Takahashi 2003). The ability of the sperm to penetrate the oocyte, an indicator of sufficient cytoplasmic components for fertilisation, also

increases with follicle size in a positive and step-wise manner (Marchal *et al.* 2002; Lucas *et al.* 2003). In cattle, the developmental competence of pre-pubertal and adult oocytes increases with follicle size from 2-3 mm compared to 4-8 mm and >8 mm (Kauffold *et al.* 2005). These findings suggest that the poor efficiency of *in vitro* embryo production (IVP) using oocytes from pre-pubertal compared to adult pigs may be due to a greater proportion of small follicles, which have reduced developmental competence in the 3-8 mm range that is typically aspirated.

A changing follicular environment appears to account for the improvement in oocyte maturation and developmental competence that is observed with increasing follicle size. The number of layers and density of cumulus cells surrounding the oocyte increases with follicle size (Yoon *et al.* 2000; Liu *et al.* 2002). Co-culture of oocytes with cumulus cells from large antral follicles improves growth and survival of smaller pre-antral follicles and oocytes (Wu *et al.* 2002). The positive effects of follicle cells on oocyte maturation and development are explained by their role in facilitating receptor-mediated gonadotropin signalling and synthesis of paracrine signalling molecules. In particular, the steroid hormones progesterone and 17β-oestradiol accumulate in the follicular fluid (FF) at concentrations several orders of magnitude higher than in blood plasma (Eiler and Nalbandov 1977) and change markedly during follicle development (Ainsworth et al., 1980). In pigs, low FF concentrations of progesterone and 17β-oestradiol correlate with the reduced developmental competence of oocytes from pre-pubertal compared with adult animals (Grupen *et al.* 2003) and in 1-2 mm follicles compared with 3-8 mm follicles in pre-pubertal animals alone (Liu *et al.* 2002).

The objectives of this chapter were (i) to determine the proportions of 3, 4 and 5-8 mm follicles on the surface of pre-pubertal and adult ovaries; (Shiozawa *et al.*) to characterise the steroid content of FF obtained from 3, 4 and 5-8 mm follicles of pre-

pubertal and adult ovaries; and (iii) to compare the developmental competence following parthenogenetic activation of oocytes from 3, 4 and 5-8 mm follicles of pre-pubertal and adult ovaries. In chapter 4 we reported that treatment with 1 mM dibutyryl cAMP (dbcAMP) for 22 h IVM increased blastocyst development of pre-pubertal, but not adult, pig oocytes by raising intra-oocyte cAMP during IVM. In this chapter we also examined the effect of 1 mM dbcAMP for the first 22h of IVM on the developmental competence of oocytes from 3, 4 and 5-8 mm follicles of pre-pubertal and adult pigs.

5.2 Experimental design

5.2.1 Experiment 1: Follicle size distribution and steroid content analysis

Follicles on the surface of pre-pubertal and adult ovaries were individually measured and the number of follicles in the 3 mm, 4 mm and 5-8 mm size cohorts was recorded. The FF from each size category was collected in preparation for steroid analysis. On average, 28 pre-pubertal ovaries were employed during each of 5 replicates with 142 ovaries analysed in total. An average of 28 adult ovaries was employed during each of 5 replicates with 135 ovaries analysed in total.

5.2.2 Experiment 2: Follicle size and parthenote embryo development

Follicles on the surface of pre-pubertal and adult ovaries were individually measured and those in the 3 mm, 4 mm and 5-8 mm size groups were aspirated. Adult cumulus-oocyte complexes (COCs) from each follicle size aspirate were matured in control conditions, whereas the pre-pubertal COCs were matured either in control conditions or with 1 mM dbcAMP for the first 22 h IVM, given the findings in Chapter 4 that dbcAMP has no effect of developmental competence of adult oocytes. Following IVM, parthenogenetic activation and IVC, the subsequent embryo development was assessed for each follicle size group, donor and treatment. Six replicates of pre-pubertal COCs

from 3 mm follicles (control n = 310, + dbcAMP treatment n = 290), 4 mm follicles (control n = 111, + dbcAMP treatment n = 99) and 5-8 mm follicles (control n = 95, + dbcAMP treatment n = 93) were matured and assessed for parthenote development. Three replicates of adult COCs from 3 mm follicles (control n = 150), 4 mm follicles (control n = 148) and 5-8 mm follicles (control n = 114) were matured and assessed for parthenote development. Generally, all three size categories were compared in the same replicate; however in the case of the 3 mm follicles it was necessary to do an additional two controlled replicates to carry out statistical comparison due to the low number of blastocysts that these follicles yield.

5.3 Results

5.3.1 Experiment 1: Follicle size distribution and steroid content analysis

Typical pre-pubertal and adult ovaries obtained from the slaughterhouse for this thesis are presented in Figure 5.1. The mean proportion and number of 3, 4 and 5-8 mm follicles occupying the surface of pre-pubertal and adult ovaries is shown in Figure 5.2. The smaller follicle sizes, 3 and 4 mm, were more abundant on the surface of both pre-pubertal and adult ovaries than the larger follicles, 5-8 mm. However, a higher proportion of 3 mm follicles populated the surface of pre-pubertal ovaries compared with adult ovaries (72 ± 4 % vs. 46 ± 4 % respectively; $P < 0.001$). Likewise, there was a lower proportion of 4 mm follicles (22 ± 3 % vs. 33 ± 3 %; $P < 0.001$) and 5-8 mm follicles (6 ± 2 vs. 21 ± 5 %; $P < 0.001$) on the surface of pre-pubertal ovaries, compared with adult ovaries.

The concentrations of progesterone and 17β-oestradiol in the FF from 3 mm, 4 mm and 5-8 mm follicles of pre-pubertal and adult ovaries is presented in Figure 5.3. The concentration of progesterone in pre-pubertal FF increased ($P < 0.001$) with follicle size

from 3 mm (60.2 ± 7.3 nmol/L) to 4 mm (81.2 ± 8.6 nmol/L) to 5-8 mm (136.8 ± 25.7 nmol/L). The concentration of progesterone in adult FF was approximately 11 fold higher than in pre-pubertal FF ($P < 0.001$), but did not differ ($P > 0.05$) between adult follicle sizes (3 mm (865.6 ± 140.9 nmol/L) to 4 mm (785.6 ± 179.1 nmol/L) to 5-8 mm (1112.6 ± 211.2 nmol/L)). The concentrations of 17β-oestradiol varied considerably among FF batches and there were no significant differences within or between pre-pubertal and adult follicles sizes ($P > 0.05$). The ratio of concentrations of progesterone to 17β-oestradiol in pre-pubertal FF is presented in Figure 5.4. The ratio of progesterone to 17β-oestradiol in pre-pubertal FF did not differ with follicle size ($P > 0.05$). In adult FF there was a trend ($P = 0.073$), for the ratio of progesterone to 17β-oestradiol to decrease with follicle size. The ratio of progesterone to 17β-oestradiol in FF from 3 mm follicles was 11 fold higher ($P = 0.008$) in adult than pre-pubertal FF.

Figure 5.1. Images of typical pre-pubertal (*i*) and adult pig ovaries (*ii*) used in this study. The white bar represents 5 mm as measured with callipers. (a) represents 3 mm follicles (b) represents large follicles 5-8 mm, (c) represents active corpora lutea (CL), (d) represents regressed CL.

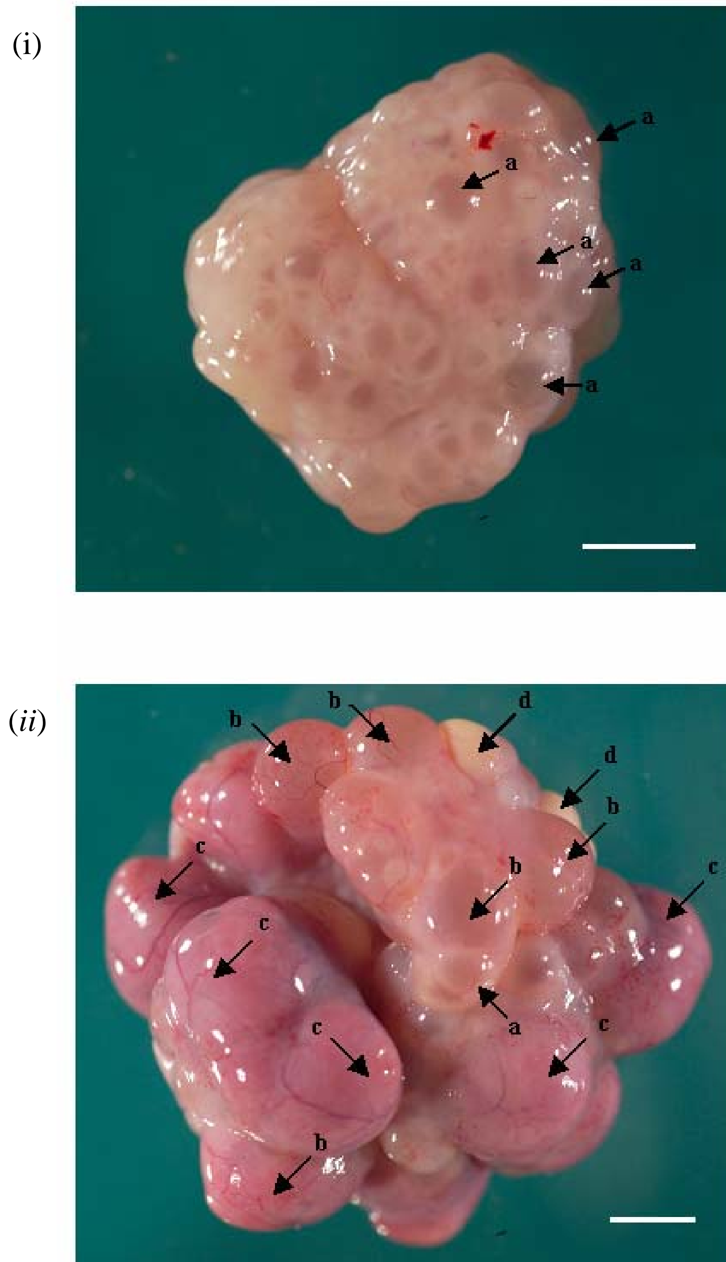


Figure 5.2. The mean percentage (*i*) and number (*ii*) of 3, 4 and 5-8 mm follicles on the surface of pre-pubertal and adult ovaries. Different superscripts indicate significant differences (P < 0.05).

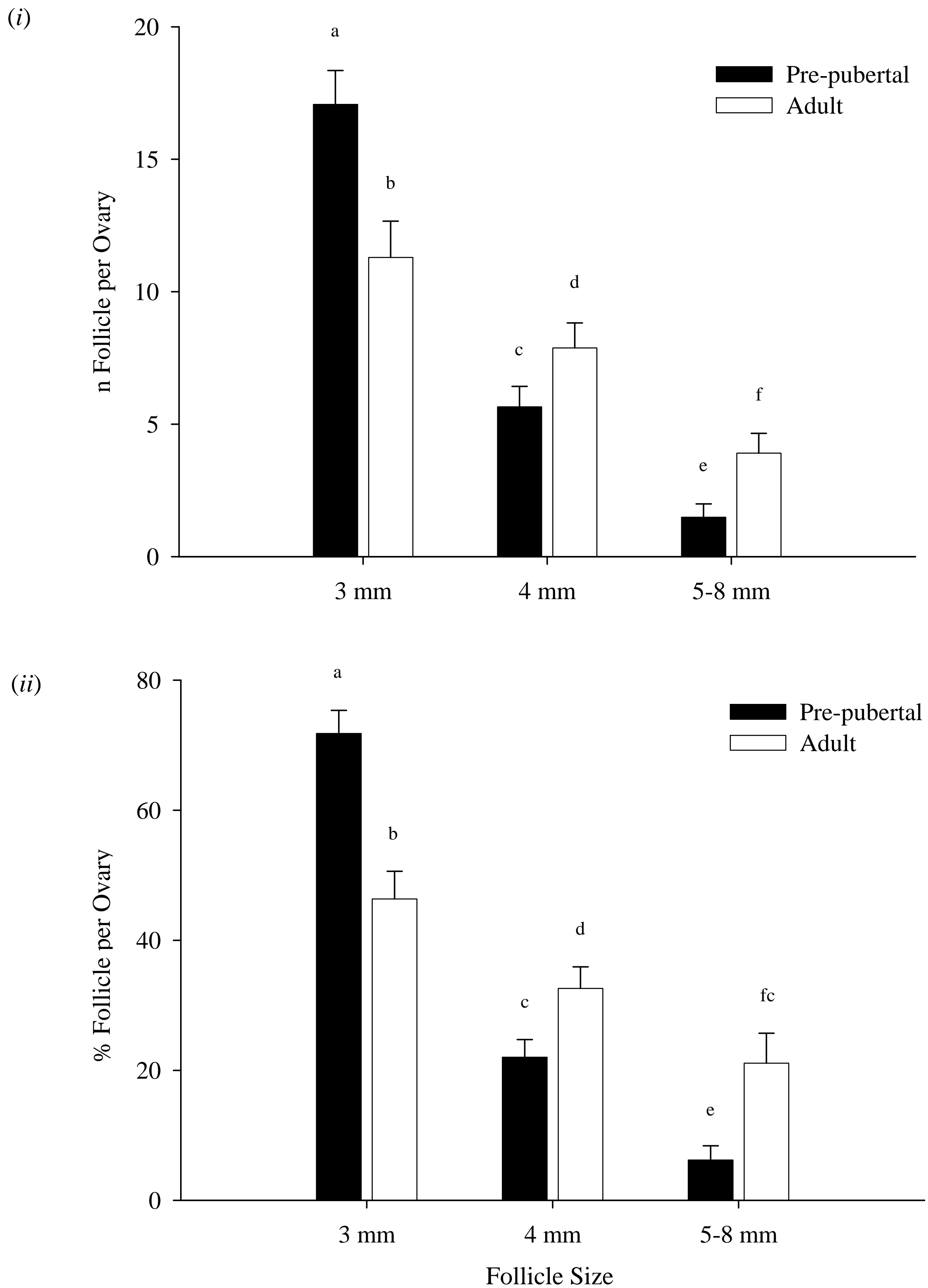


Figure 5.3 The concentrations of (*i*) progesterone (P4) and (*ii*) 17β-oestradiol (E2) in FF aspirated from 3, 4 and 5-8 mm pre-pubertal and adult ovarian follicles. Different superscripts indicate significant differences ($P < 0.05$).

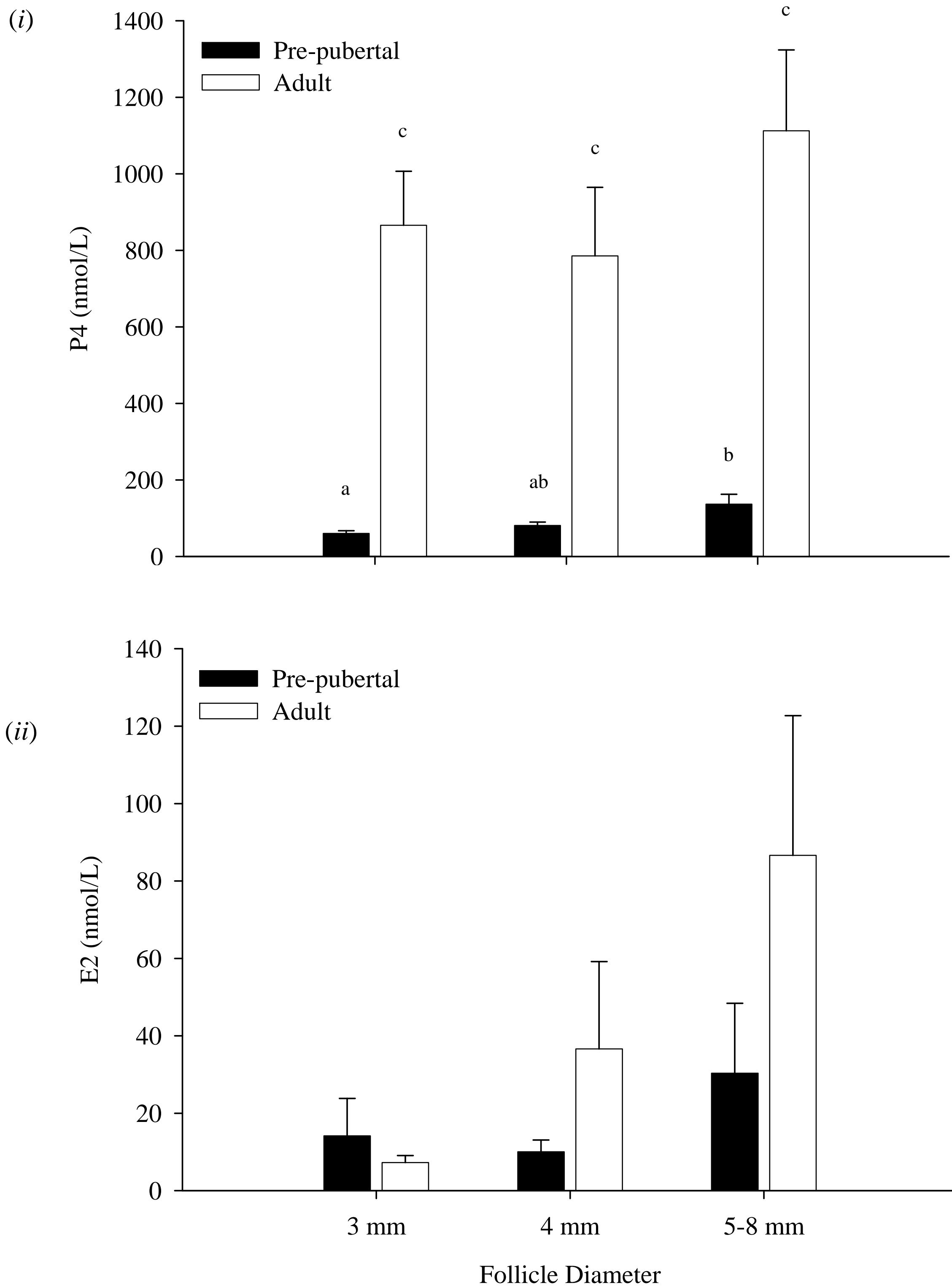
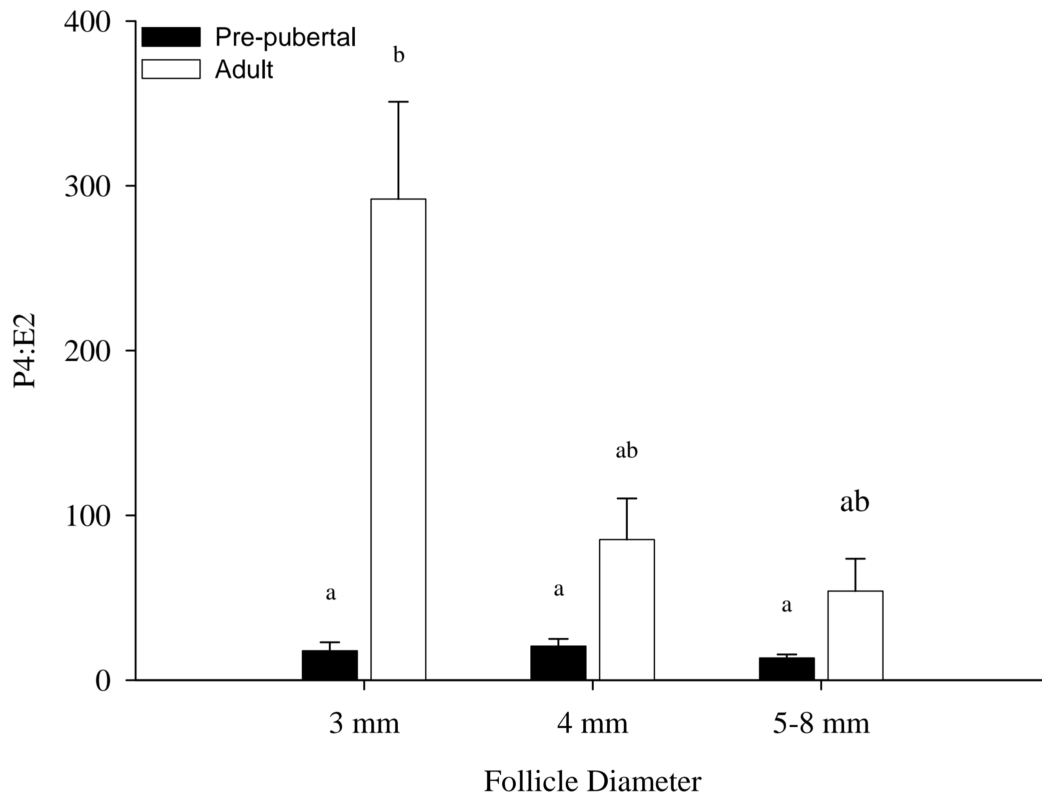


Figure 5.4 The molar ratio of progesterone to 17β-oestradiol (P4:E2) in FF aspirated from 3, 4 and 5-8 mm pre-pubertal and adult ovarian follicles. Different superscripts indicate significant differences ($P < 0.05$).



5.3.2 Experiment 2: Follicle size and parthenote embryo development

Maturation rates and subsequent embryo development rates for pre-pubertal and adult oocytes from 3 mm, 4 mm and 5-8 mm follicles are presented in Table 5.2. Rates of maturation and embryo cleavage were not different ($P > 0.05$) between pre-pubertal and adult oocytes from 3 mm, 4 mm and 5-8 mm follicles. A higher percentage of pre-pubertal oocytes from 3 mm follicles occupied the < 8-cell embryo development stage compared with those from 5-8 mm follicles (54 % vs. 23 %, $P = 0.029$). However, there were no differences between pre-pubertal and adult oocytes or follicle size groups in the number of embryos that were at the morula stage at the time of assessment. Adult oocytes displayed similar development to blastocyst from all of the three follicle sizes ($P > 0.05$). Blastocyst development from pre-pubertal oocytes improved with increasing follicle size ($P < 0.001$) from 3 mm (17 %) to 4 mm (36 %) to 5-8 mm (55 %). There was no difference in blastocyst development between pre-pubertal oocytes from 5-8 mm follicles and adult oocytes from all three follicle sizes ($P > 0.05$). Embryo development to 8-cell and morula in Table 5.1 is expressed relative to the number of embryos that had cleaved. The effects on development rates to 8-cell, morula and blastocyst were similar whether expressed relative to the total number of embryos activated, or the number that cleaved. Both data analyses are shown for blastocyst development (Table 5.1). There was a nearly two-fold increase in the mean cell number in blastocysts developed from pre-pubertal oocytes from 3 mm follicles compared with 5-8 mm follicles (24 vs. 42 cells; $P= 0.045$). The mean blastocyst cell number of adult parthenotes was similar (50 cells; $P > 0.05$) amongst all three follicle sizes studied and around 2-fold ($P= 0.021$) that of pre-pubertal blastocysts from 3 mm and 4 mm follicles.

The developmental results for pre-pubertal oocytes matured in the presence of dbcAMP are also presented in Table 5.1 and will be discussed here in context to the control

results (described above) for pre-pubertal and adult oocytes from the three different follicle sizes. Blastocyst development from 3 mm pre-pubertal oocytes was improved 1.5 fold when oocytes were matured for the first 22h IVM with 1 mM dbcAMP (17 % to 29 %, $P = 0.043$). Maturation in the presence of 1mM dbcAMP for the first 22 h IVM had no effect on pre-pubertal oocytes from 4 and 5 mm follicles ($P > 0.05$). Despite the improvement, dbcAMP treated pre-pubertal oocytes from 3 mm follicles still displayed lower developmental competence compared with control matured 5 mm pre-pubertal oocytes ($P = 0.003$) or any of the adult oocytes ($P = 0.003$). The mean blastocyst cell number of pre-pubertal oocytes was similar ($P > 0.05$) despite IVM in the presence or absence of dbcAMP.

Table 5.1 Comparison of developmental competence between oocytes from 3, 4 and 5-8 mm follicles of pre-pubertal oocytes, with and without dbcAMP treatment, and Adult oocytes following parthenogenetic activation.

Follicle Size Groups	**n**	**% MII**	**% Cleaved**	**% < 8 Cell**	**% Morula**	**% Blastocyst (per total)**	**% Blastocyst (per cleaved)**	**Mean cell no. (Day 7 blastocyst)**
Pre-pubertal Oocytes								
3 mm	310	89 ± 2 ^{a}	85 ± 4 ^{a}	54 ± 8 ^{ac}	15 ± 5 ^{a}	14 ± 3 ^{a}	17 ± 4 ^{a}	24 ± 6 ^{a}
4 mm	111	96 ± 2 ^{a}	85 ± 5 ^{a}	30 ± 6 ^{ab}	15 ± 7 ^{a}	31 ± 6 ^{bc}	37 ± 7 ^{b}	30 ± 11 ^{ab}
5-8 mm	95	96 ± 0 ^{a}	82 ± 13 ^{a}	23 ± 4 ^{b}	10 ± 3 ^{a}	44 ± 8 ^{c}	55 ± 6 ^{c}	42 ± 10 ^{bd}
3 mm + dbcAMP	290	89 ± 2 ^{a}	82 ± 3 ^{a}	45 ± 5 ^{ac}	10 ± 4 ^{a}	24 ± 4 ^{b}	29 ± 4 ^{b}	20 ± 5 ^{ab}
4 mm + dbcAMP	99	95 ± 2 ^{a}	88 ± 4 ^{a}	51 ± 7 ^{ac}	7 ± 3 ^{a}	24 ± 8 ^{ab}	27 ± 8 ^{ab}	25 ± 7 ^{abc}
5-8 mm + dbcAMP	93	96 ± 1 ^{a}	87 ± 6 ^{a}	26 ± 2 ^{b}	12 ± 8 ^{a}	46 ± 4 ^{c}	53 ± 6 ^{c}	28 ± 8 ^{abc}
Adult Oocytes								
3 mm	150	92 ± 2 ^{a}	79 ± 6 ^{a}	24 ± 2 ^{b}	7 ± 2 ^{a}	41 ± 2 ^{c}	53 ± 4 ^{c}	46 ± 11 ^{cd}
4 mm	148	97 ± 1 ^{a}	77 ± 8 ^{a}	28 ± 6 ^{bc}	5 ± 1 ^{a}	42 ± 5 ^{c}	55 ± 7 ^{c}	52 ± 11 ^{d}
5-8 mm	114	96 ± 1 ^{a}	84 ± 7 ^{a}	32 ± 6 ^{bc}	2 ± 1 ^{a}	47 ± 3 ^{c}	57 ± 6 ^{c}	51 ± 11 ^{d}

[abcd] Within columns, values with different superscripts indicate significant differences ($P < 0.05$).

[1]Values expressed as mean ± S.E.M.

[2]Data are expressed as mean percentage of cleaved.

[3]Data are expressed as mean percentage of total.

5.4 Discussion

The experiments presented in this chapter describe and compare the distribution, composition and oocyte developmental competence of antral follicle size categories within the common 3-8 mm follicle aspiration range on the surface of pre-pubertal and adult pig ovaries. The results demonstrate that pre-pubertal ovaries contain a greater proportion of small, 3 mm, follicle sizes compared with adult ovaries. The developmental competence of pre-pubertal porcine oocytes, but not adult oocytes, was shown to increase in parallel with follicle size, with oocytes from 3 mm follicles resulting in the lowest blastocyst rates. This is in agreement with previous studies in the cow and pig, which also report that blastocyst formation of pre-pubertal oocytes increases with increasing follicle size (Marchal *et al.* 2002; Kauffold *et al.* 2005). This chapter extends these findings by examining and comparing the developmental competence of oocytes from 3 mm, 4 mm and 5-8 mm follicles of pre-pubertal and adult pig ovaries. The rate of blastocyst development for adult oocytes did not differ significantly between the three follicle sizes, suggesting that oocytes from these follicles have acquired the same capacity to attain full developmental potential following final maturation. This finding contrasts with previous findings reported for adult cow oocytes, where blastocyst formation also increased with follicle size (Kauffold *et al.* 2005). Our findings indicate that poor efficiency of *in vitro* embryo production from pre-pubertal oocytes compared with adult oocytes is due to the low developmental competence of pre-pubertal oocytes from 3 and 4 mm follicles, and a far greater proportion of 3 mm follicles on the surface of pre-pubertal compared to adult ovaries.

When pre-pubertal oocytes from different sized follicles were matured in the presence of dbcAMP, rates of blastocyst formation were only increased significantly for oocytes derived from 3 mm follicles. In contrast, the blastocyst development of pre-pubertal

oocytes from 4 and 5-8 mm follicles was not affected by the dbcAMP treatment. This result suggests that the stimulatory effect of dbcAMP on the development of pre-pubertal oocytes reported previously in chapter 4 was the result of its action on oocytes from 3 mm follicles, which presumably comprised the majority of the 3-8 mm follicles aspirated for IVM in that study. In chapter 4 we reported that pre-pubertal oocytes have a reduced cAMP content compared with adult oocytes during IVM, and that this could be increased with dbcAMP treatment for the first 22 h of IVM. In light of the findings reported here, this suggests that pre-pubertal oocytes from 3 mm follicles are likely to have a reduced ability to accumulate cAMP during IVM compared with oocytes from larger follicles. This interpretation is further supported by a previous study in pre-pubertal pigs, where the ability of granulosa cells to stimulate intra-oocyte cAMP concentration increased progressively with follicle size (Mattioli *et al.* 1994). Treatment with dbcAMP in the present study could only partially increase the developmental competence of pre-pubertal oocytes from 3 mm follicles and did not improve development of oocytes from 4 mm follicles. This may indicate that cAMP-related deficiencies only affect oocytes within 3 mm follicles and that other follicular-size dependent factor (s), in addition to cAMP, are required for complete oocyte maturation.

The mean cell number of blastocysts from all of the adult oocytes and pre-pubertal oocytes from 5-8 mm follicles was higher than that for pre-pubertal oocytes from 3 and 4 mm follicles, suggesting embryo quality also increases with follicle size in pre-pubertal pigs. Our finding is in agreement with previous reports on the mean cell number in porcine parthenogenetic blastocysts (between 40 to 60 cells) and extends these findings by examining cell numbers in blastocysts resulting from different follicle sizes on pre-pubertal and adult pig ovaries (Cui 2004, Cui 2005 a & b, Booth et al., 2005; Algriany et al., 2004). Treatment of oocytes with dbcAMP did not change the

total blastocyst cell numbers, suggesting that factors other than cAMP levels during IVM contribute to this indicator of blastocyst quality. However, this result should be interpreted with caution as it could also be a remnant of the parthenogenetic process, with lower cell numbers observed in porcine embryos created via parthenogenetic activation compared to those created by IVF (60 vs. 90 cells respectively) (Koo et al., 2000).

While the progesterone concentration of pubertal FF increased with follicle size, it was still approximately 11-fold lower than in adult follicles even at its greatest concentration in 5-8 mm follicles. This finding supports a previous study (Grupen *et al.* 2003), which reported progesterone to be nearly double in the FF (3-8 mm follicles) of adult compared to pre-pubertal pigs. The 17β-oestradiol concentration in FF was not significantly different between follicle size and/or donor age. This contrasts with previous reports that the 17β-oestradiol concentration was around 3 fold higher in the FF of adult compared to pre-pubertal pigs (Grupen *et al.* 2003). The ratio of progesterone to 17β-oestradiol was 30-fold higher in adult compared to pre-pubertal FF from 3 mm follicles, but was similar between FF from 4 mm and 5 mm follicles. These results may reflect differences in the number of gonadotropin receptors present or the steroidogenic activity of follicle somatic cells of different follicle sizes and donor ages. However, intra-ovarian diffusion of steroids from adjacent steroidogenic components, such as corpora lutea and possibly large follicles or interstitial cells, cannot be excluded and may be responsible for the higher progesterone concentrations in follicles of all sizes from adult ovaries. Low fertility and increased embryonic mortality *in vivo* along with reduced rates of blastocyst formation *in vitro* have also been reported for pigs at first compared to third oestrous (Archibong *et al.* 1987; Menino *et al.* 1989). Intra-ovarian diffusion of steroids *in vivo* seems a likely explanation for the improved embryo

development observed in adult pigs of multiple oestrous cycles, with an increased presence of corpora lutea resulting in exposure of pre-ovulatory oocytes to higher progesterone concentrations.

In conclusion, this chapter demonstrates that the poor efficiency of embryo IVP from pre-pubertal pig oocytes is due to presence of a large population of 3 mm follicles on the surface of pre-pubertal ovaries, which contain oocytes of low developmental potential. Pre-pubertal oocytes from 3 mm follicles have not attained the developmental potential that is acquired with increasing follicle size in pre-pubertal pig and already exists in 3-8 mm follicles of adult pigs. In addition, the steroidal differences between pre-pubertal and adult donors and between pre-pubertal follicle sizes indicate that the 3 mm pre-pubertal follicle contains a more immature growth environment while larger pre-pubertal follicles and adult follicles are closer to ovulatory maturity. The improved, but not completely rescued, blastocyst development of 3 mm pre-pubertal oocytes following IVM in the presence of dbcAMP, suggests that final oocyte maturation for full acquisition of developmental competence involves both cAMP mediated and other as yet unknown processes.

Chapter 6

Effect of follicle size and dbcAMP on the cAMP content of pre-pubertal oocytes and COCs

6 Effect of follicle size and dbcAMP on the cAMP content of pre-pubertal oocytes and COCs

6.1 Introduction

The experiments in chapter 4 revealed that the reduced developmental capacity of pre-pubertal compared to adult pig oocytes appears to be a consequence of their reduced ability to accumulate cAMP in response to FSH supplied during *in vitro* maturation (IVM). Chapter 5 demonstrated that the low developmental competence of pre-pubertal compared with adult pig oocytes is associated with a greater proportion of 3 mm follicles in the standard 3-8 mm follicle aspiration range. In particular, pre-pubertal oocytes from 3 mm follicles display a reduced blastocyst development compared with those from 4 mm and 5-8 mm follicles and adult oocytes from all follicle sizes aspirated. Maturation in the presence of dubutyryl cAMP (dbcAMP) had a positive effect on blastocyst development of pre-pubertal oocytes from 3 mm follicles, but not 4 and 5-8 mm follicles or adult oocytes from any follicle size. These findings suggest that the mechanism(s) for increasing intra-oocyte cAMP during IVM are deficient in pre-pubertal cumulus-oocyte complexes (COCs) from 3 mm follicles. As such, pre-pubertal COCs from 3 mm and 5-8 mm follicles were used as a model to further examine low versus high developmental competence.

The majority of intra-oocyte cAMP is synthesised in the cumulus cells and enters the oocyte via gap junctions (Schultz *et al.* 1983a; Racowsky 1985). Time dependent changes in intra-oocyte cAMP have been reported for pre-pubertal pig COCs matured *in vivo* (Mattioli *et al.* 1994) and *in vitro* (Shimada and Terada 2002a). These changes not only appear to be important for GVBD (Racowsky 1985; Mattioli *et al.* 1994), but also for further meiotic and possibly cytoplasmic maturation (Shimada and Terada 2002a).

The results of experiments presented in Chapter 4 of this thesis were consistent with those of a previous study, which observed no increase in the intra-oocyte cAMP content of COCs during IVM (Mattioli *et al.* 1994). In contrast, Shimada and Terada (2002a) reported that the intra-oocyte cAMP concentration of pooled pre-pubertal COCs from 3-8 mm follicles reaches a peak concentration by 8 h IVM, and then decreases to reach basal levels by 32 h, where it remains until the end of maturation. Furthermore, Shimada et al. (2002a) used forskolin, IBMX, PI 3-kinase inhibitors and PKC inhibitors to maintain high intra-oocyte cAMP levels and demonstrated that a drop in cAMP post MI is necessary for progression to MII (Shimada and Terada 2002a). These three studies provide conflicting reports on the pattern of cAMP production during IVM of pre-pubertal pig oocytes. Moreover, none of these studies have examined the relationship between intra-oocyte cAMP during IVM and follicle size.

The aim of this chapter was to compare the cAMP content of oocytes and COCs, and cumulus expansion from 3 mm and 5-8 mm follicles during IVM. The response to dbcAMP treatment for the first 22 h of IVM was also assessed.

6.2 Experimental design

6.2.1 Experiment 1: Oocyte cAMP content

Pre-pubertal COCs from each follicle size group were matured in control conditions or with 1 mM dbcAMP for the first 22 h IVM. At 0 h, 6 h, 11 h and 22 h IVM a proportion of COCs from each group were denuded and the zona-pellucida was removed to measure intra-oocyte cAMP. Four replicates of COC-derived oocytes from 3 mm follicles (control n = 407, + dbcAMP treatment n = 451) and 5-8 mm follicles (control n = 385, + dbcAMP treatment n = 364) were assessed for cAMP content. Both follicle sizes and all time points were examined in the same experiment.

6.2.2 Experiment 2: COC cAMP content

Pre-pubertal COCs from each follicle size group were matured in control conditions or with 1 mM dbcAMP for the first 22 h of IVM. A proportion of oocytes from each group were removed at 0 h, 6 h, 11 h and 22 h IVM for cAMP measurement. Where possible COC and oocytes were collected for cAMP analysis on the same day, however this was dependent on the number of COCs available for the experiment. At least 4 replicates of intact pre-pubertal COCs from 3 mm follicles (control n = 282, + dbcAMP treatment n = 284) and 5-8 mm follicles (control n = 275, + dbcAMP treatment n = 257) were assessed for cAMP content, both follicle sizes and all time points were examined in the same experiment.

6.2.3 Experiment 3: Cumulus expansion

Pre-pubertal COCs from each follicle size group were matured in control conditions or with 1 mM dbcAMP for the first 22 h IVM and then assessed for cumulus expansion at 11 h and 22 h IVM. At 11 h and 22 h IVM, five replicates of COCs from 3 mm follicles (control n = 175, + dbcAMP treatment n = 165) and four replicates of 5-8 mm follicles (control n = 183, + dbcAMP treatment n = 184) were assessed for cumulus expansion.

6.3 Results

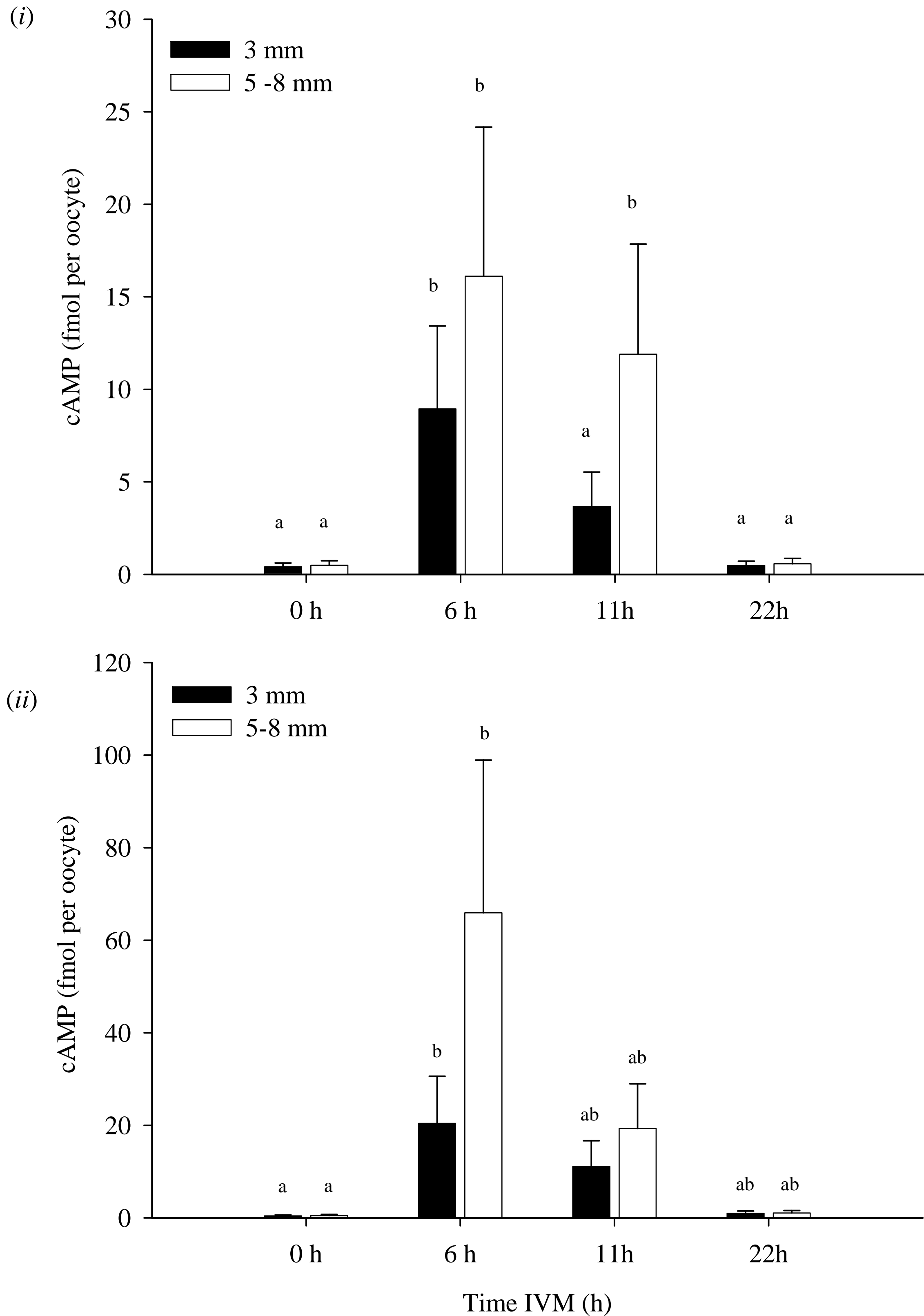
6.3.1 Oocyte cAMP content

The time-dependent changes in cAMP concentration of zona-free oocytes from 3 mm and 5-8 mm follicles are presented in Figure 6.1. Oocytes recovered from 3 mm and 5-8 mm follicles contained similar concentrations of cAMP at 0 h IVM (0.4 ± 0.2 fmol vs. 0.5 ± 0.2 fmol respectively, $P > 0.05$). Following 6 h IVM, there was a significant increase ($P < 0.05$) in the cAMP content of both oocytes from 3 mm and 5-8 mm

follicles (8.9 ± 2.7 fmol and 16.11 ± 6.9 fmol respectively). By 11 h IVM, while the cAMP concentration of oocytes from 3 mm follicles had decreased (3.6 ± 1.8 fmol; P = 0.069), the cAMP content of oocytes from 5-8 mm follicles remained at a similar level (11.9 ± 5.9 fmol) and was significantly higher than in oocytes from 3 mm follicles (P = 0.05). By 22 h IVM, the cAMP concentrations of oocytes from 5-8 mm follicles had decreased again from 11 h (P = 0.036), and no significant differences were observed between oocytes from the two follicle size groups (0.5 ± 0.2 fmol vs. 0.6 ± 0.3 fmol respectively; P > 0.05).

In the presence of dbcAMP, the cAMP concentration of oocytes from 3 mm and 5-8 mm follicles followed a similar, yet amplified, pattern of cAMP changes compared to oocytes matured in control conditions. In the presence of dbcAMP, the difference in cAMP concentration between oocytes from 3 mm and 5-8 mm follicles at 11 h IVM no longer existed (11.1 ± 5.6 fmol vs. 19.3 ± 9.7 fmol, P > 0.05). By 22 h IVM, the cAMP content of oocytes from 3 mm and 5-8 mm follicles had both decreased to basal levels (1 ± 0.5 fmol vs. 1 ± 0.05 fmol respectively).

Figure 6.1. Cyclic AMP concentrations of pre-pubertal oocytes from 3 mm and 5-8 mm follicles at 0, 6, 11 and 22h IVM in control conditions (*i*) or with 1 mM dbcAMP (*ii*). Different superscripts indicate significant differences in cAMP concentrations ($P < 0.05$).

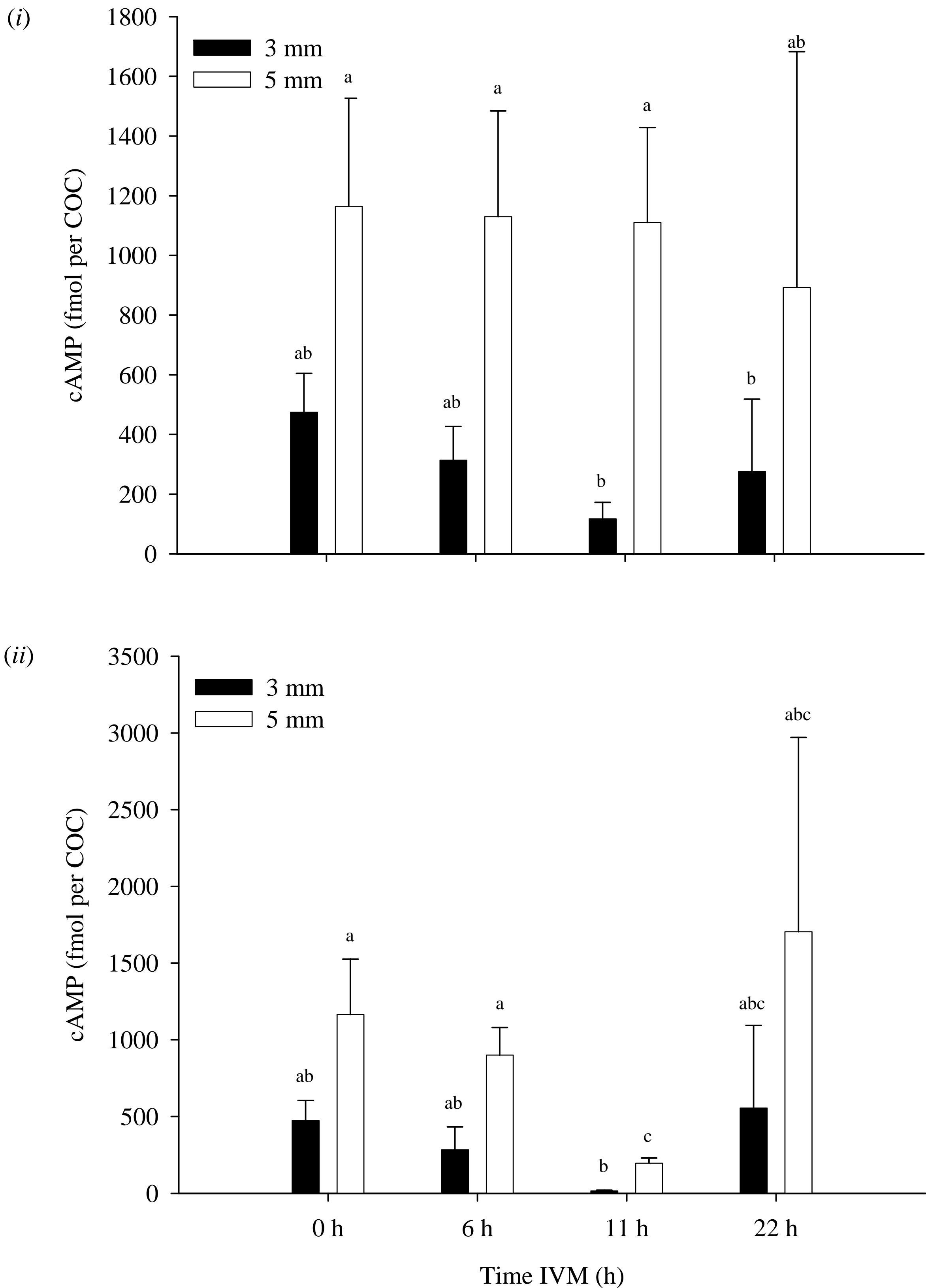


6.3.2 COC cAMP content

The time-dependent changes in cAMP concentration of intact COCs from 3 mm and 5-8 mm follicles are presented in Figure 6.2. The cAMP concentration of COCs in this study was 50-150 fold higher than for ZF oocytes. COCs from 3 and 5-8 mm follicles contained similar concentrations of cAMP at 0 h (474.4 ± 130.6 fmol vs. 1164.7 ± 361.5 fmol respectively, P > 0.05) and 6 h IVM (313.5 ± 113.5 fmol vs. 1129.4 ± 354.6 fmol respectively, P > 0.05). However, by 11 h IVM the cAMP concentration of COCs from 3 mm follicles was 11 fold lower than in COCs from 5-8 mm follicles. At 22 h IVM no significant difference existed between cAMP concentration of COCs from 3 mm and 5-8 mm follicles.

In the presence of dbcAMP, the cAMP concentration of COCs from 3 and 5-8 mm follicles did not markedly change during IVM and was of similar magnitude to COCs matured under control conditions. However, at 11 h IVM the cAMP concentration of COCs from 3 mm follicles was around 13 fold lower than those from 5-8 mm follicles (15.1 ± 4.9 fmol vs. 196.2 ± 33.3 fmol respectively, P = 0.029). By 22 h IVM, the cAMP concentrations of COCs from both 3 mm and 5-8 mm follicles occupied levels similar to 0h IVM, and no significant differences were observed between the two groups.

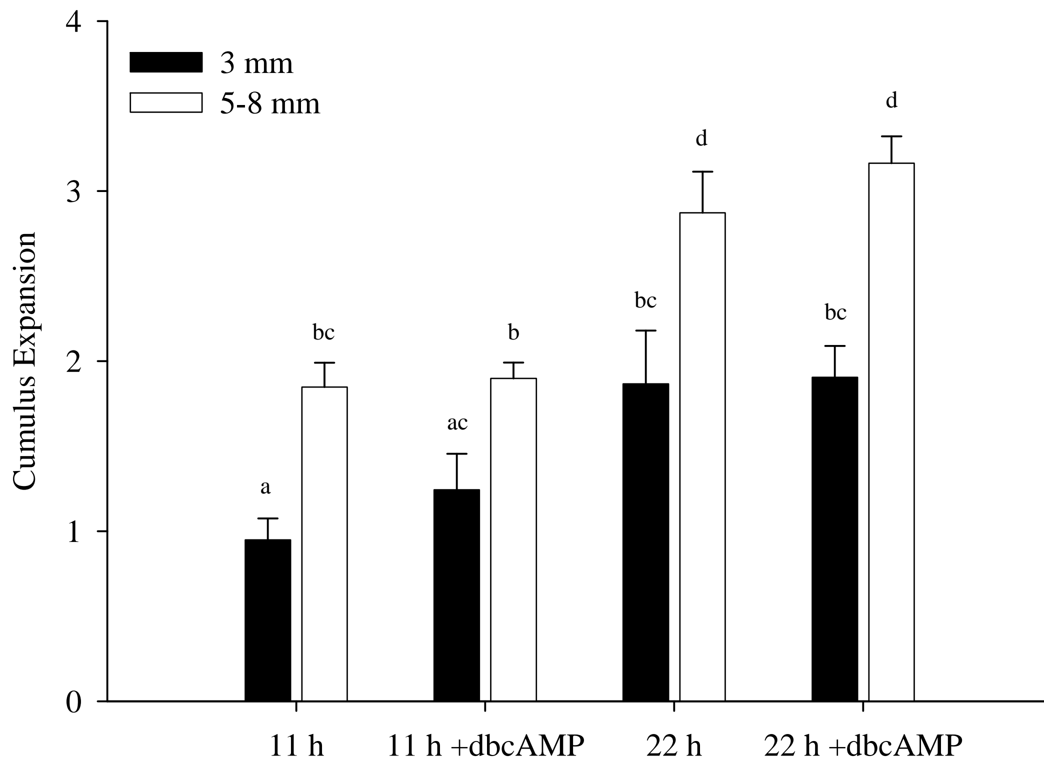
Figure 6.2. Cyclic AMP concentrations of pre-pubertal COCs from 3 mm and 5-8 mm follicles at 0, 6, 11 and 22h IVM in control conditions (*i*) or with 1 mM dbcAMP (*ii*). Different superscripts indicate significant differences in cAMP concentrations ($P < 0.05$).



6.3.3 Cumulus expansion

The cumulus expansion of COCs from 3 mm and 5-8 mm follicles is presented in Figure 6.3. By 11 h of control IVM COCs from 5-8 mm follicles displayed significantly increased cumulus expansion compared to those from 3 mm follicles (1.8 ± 0.1 vs. 1.0 ± 0.1 respectively, P = 0.003). Between 11 and 22 h control IVM, there was a significant increase in the degree of cumulus expansion of COCs from 3 mm (P = 0.043) and 5-8 mm (P = 0.014) follicles, yet COCs from 5-8 mm follicles still displayed a greater degree of cumulus expansion compared to those from 3 mm follicles (2.9 ± 0.2 vs. 1.9 ± 0.3 respectively, P = 0.042). When matured in the presence of dbcAMP, the degree of cumulus expansion was still greater in COCs from 5-8 mm compared with 3 mm follicles at both 11 (1.9 ± 0.1 vs. 1.2 ±0.2, P = 0.036) and 22 h IVM (3.2 ± 0.16 vs. 1.9 ± 0.2, P = 0.011). The difference in cumulus expansion between COCs from 3 mm and 5-8 mm was more pronounced with time regardless of maturation conditions. Dibutyryl cAMP had no significant influence on cumulus expansion in either follicle size class or time of culture.

Figure 6.3. Cumulus expansion of pre-pubertal COCs from 3 mm and 5-8 mm follicles at 11 h and 22 h IVM in control or dbcAMP treatment conditions. Different superscripts indicate significant differences in cumulus expansion (P < 0.05).



6.4 Discussion

This chapter describes the time-dependent pattern of cAMP concentrations in oocytes and intact COCs from 3 mm and 5-8 mm follicles of pre-pubertal pigs during IVM. The findings presented here agree with and extend other reports on the time-dependent pattern of cAMP during maturation of pre-pubertal pig oocytes and COCs pooled from 3-8 mm follicles (Racowsky 1985; Mattioli *et al.* 1994; Shimada and Terada 2002a). The cAMP concentration of oocytes from 5-8 mm follicles was at least 3 fold higher compared with oocytes from 3 mm follicles following 11 h of IVM, confirming that pre-pubertal COCs from 3 mm follicles have a reduced ability to accumulate intra-oocyte cAMP in response to FSH in the IVM medium. Following 11 h IVM in the presence of dbcAMP, the cAMP content of oocytes from 3 mm follicles was similar to that of oocytes from 5-8 mm follicles. These results add further support to the interpretation that the low developmental competence of pre-pubertal oocytes from 3 mm follicles relates to reduced levels of intra-oocyte cAMP.

The cAMP concentration of intact COCs from 5-8 mm follicles was 11 fold higher than in COCs from 3 mm follicles at 11 h. Since the cumulus layer is the main source of cAMP synthesis in the COC (Schultz *et al.* 1983a; Racowsky 1985), the present result suggests that the cumulus layer of COCs from 3 mm follicles cannot produce or maintain cAMP to the same level as that for COCs from 5-8 mm follicles during IVM and that this directly correlates with intra-oocyte cAMP. Another explanation could be that there are twice as many cumulus cells in the 5-8 mm COCs compared with the 3 mm COCs. In the presence of dbcAMP, the difference in cAMP content between COCs from 3 mm and 5-8 mm follicles at 11 h of IVM remains similar to that observed in control conditions. The cross reactivity between dbcAMP and the cAMP antibody employed in this assay was around 1%, suggesting that these results represent the actual

cAMP content of the COC. In addition, the low values observed at 0 h for dbcAMP treated oocytes provides confidence that there is minimal residual cAMP being measured. Since maturation in the presence of dbcAMP does not increase the cAMP content of the whole COC, but only increases the intra-oocyte cAMP content of COCs from 3 mm follicles, it appears dbcAMP may be having its effect in a different manner than by simply increasing the cAMP levels in the cumulus layer.

One explanation could be that dbcAMP increases intra-oocyte cAMP by increasing GJC between cumulus-cumulus and/or cumulus-oocyte cell types. Dibutyryl cAMP has been previously reported to increase the intercellular GJC between somatic cell types in the sheep and mouse (Eppig and Ward-Bailey 1982; Grazul-Bilska *et al.* 2001) and can modulate number, size and the distribution of gap junctions in adrenal and rat prostate tumour cells (Murray *et al.* 1998). Along this line of reasoning, the dbcAMP-mediated improvement in blastocyst development of pre-pubertal oocytes from 3 mm follicles and the high developmental competence of COCs from 5-8 mm follicles may be explained by an increased number and/or functionality of GJC within the COC.

On comparison of the cumulus expansion of COCs from 3 mm and 5-8 mm follicles during IVM, COCs from 3 mm follicles displayed lower cumulus expansion than the COCs from 5-8 mm follicles at both 11 h and 22 h. Cumulus expansion increases throughout IVM, with the degree of cumulus expansion generally taken to indicate the extent of oocyte cytoplasmic maturation and preparation for successful fertilisation and embryo development. This result suggests that the COCs from 3 mm follicles are not as mature as COCs from 5-8 mm follicles. There are conflicting reports as to whether cumulus expansion is an indicator of GJ occlusion in the pig (Motlik *et al.* 1986; Isobe *et al.* 1998; Mori *et al.* 2000; Isobe and Terada 2001). Again, there is the possibility that the greater expansion of 5-8 mm COCs is simply a reflection of a greater number of

cumulus cells, giving the appearance of more expansion. Two previous studies in the pig have reported that cumulus expansion is not indicative of gap junction closure in the pig (Suzuki *et al.* 2000; Sato and Yokoo 2005). In this chapter, dbcAMP had no effect on cumulus expansion of COCs from 3 mm or 5-8 mm follicles and while this result is in agreement with those of chapter 4, it contrasts with a mouse study that reported that 0.25 mM dbcAMP inhibits cumulus expansion (Eppig and Ward-Bailey 1982). These results support poor cumulus expansion as being an indicator of subsequent poor developmental ability (Hashimoto *et al.* 1998; Yokoo and Sato 2004), yet these were not correlated previously in chapter 4 of this thesis.

In conclusion, this chapter demonstrates that COCs from 3 mm follicles accumulate less intra-oocyte and inter-COC cAMP and display lower cumulus expansion in response to FSH in the IVM media than COCs from 5-8 mm follicles. While treatment with dbcAMP increased the cAMP content of oocytes from 3 mm follicles, it had no effect on the cAMP content or cumulus expansion of the whole COC. These results confirm that pre-pubertal oocytes and COCs from 3 mm follicles are unable to accumulate sufficient cAMP during IVM as efficiently as COCs from 5-8 mm follicles, and hence are less able to complete the cAMP-mediated maturation step(s) that are required for complete maturation, fertilisation and development. The positive effect of dbcAMP on the cAMP content of oocytes from 3 mm follicles, but not the entire COC, may be a result of enhanced cAMP transport or synthesis within the COC, or reduced degradation of intra-oocyte cAMP. These possibilities are examined in the next chapter.

Chapter 7

Effect of follicle size and dbcAMP on cumulus cell-oocyte GJC during IVM

7 Effect of follicle size and dbcAMP on cumulus cell-oocyte GJC during IVM

7.1 Introduction

Experiments in chapter 5 determined that pre-pubertal pig ovaries have a greater proportion of 3 mm follicles on their surface compared with adult ovaries. This is relevant as pre-pubertal oocytes from 3 mm follicles display reduced developmental competence compared with pre-pubertal oocytes from 5-8 mm follicles and adult oocytes from 3-8 mm follicles. In chapter 6, the reduced developmental capacity of pre-pubertal oocytes from 3 mm compared with 5-8 mm follicles was correlated with significantly lower intra-oocyte and intra- cumulus-oocyte complex (COC) cAMP concentrations during *in vitro* maturation (IVM). Treatment with dibutyryl cAMP (dbcAMP) significantly increased the cAMP level of 3 mm pre-pubertal oocytes, but had no effect on the cAMP content of the whole COC. One possibility, which would explain this result, is that dbcAMP increases intra-oocyte cAMP not simply by increasing the cAMP concentration of the whole COC and may have been achieved by enhancing pathways of cAMP transfer or synthesis, or by blocking degradation of cAMP or both.

The majority of intra-oocyte cAMP is synthesised in the cumulus cells of the COC by adenylate cyclase activity, in response to the gonadotropins supplied during IVM, and is transferred via gap junctions to the oocyte (Schultz *et al.* 1983b; Racowsky 1985; Webb *et al.* 2002). Heterologous gap junction communication (GJC) between the granulosa cells and the oocyte occurs via an extensive network of connexin (Cx) channels, and is essential for follicle and oocyte development (Buccione *et al.* 1990). Cx 43 and Cx 37 appear the most relevant connexins for investigations into heterologous GJC between the cumulus cells and the oocyte, with Cx 43 expressed both on granulosa cells and the oocyte and Cx 37 expressed in oocytes at all stages of folliculogenesis (Risek *et al.*

1990; Simon *et al.* 1997; Granot *et al.* 2002). GJC coupling between the oocyte and its cumulus cells first appears as primordial follicles form and increases during folliculogenesis (Mitchell and Burghardt 1986; Eppig 1991; Eppig *et al.* 1996). Gonadotropin levels also change throughout follicle growth and development, with FSH essential for the growth of small follicles (< 2mm) and increased LH necessary for follicle growth beyond 2 mm in the pig (Driancourt MA 1995). The changing levels of FSH and LH are important for the production of steroids by the granulosa cells, which facilitate further follicle development. In the pre-ovulatory follicle, LH and FSH stimulate progesterone production and progesterone receptor (PR) synthesis in cumulus cells (Shimada and Terada 2002b; Yamashita *et al.* 2003), a high level of which leads to decreased expression of the Cx 43 gap junction protein in the rat and pig (Granot and Dekel 1994; Shimada and Terada 2002b). LH appears to promote oocyte maturation by interrupting GJC between the oocyte and its surrounding follicle cells, reducing transfer of meiotic inhibitory substances, such as cAMP (Gilula *et al.* 1978; Larsen *et al.* 1986; Motlik *et al.* 1986; Larsen *et al.* 1987; Granot and Dekel 1994). Cyclic AMP and dbcAMP can increase the number and/or maintenance of GJC between various cell types in a number of tissues and species (Eppig and Ward-Bailey 1982; Ghosh and Singh 1997; Murray *et al.* 1998; Cruciani and Mikalsen 2002). Together these findings suggest that the dbcAMP mediated improvement in blastocyst development of pre-pubertal oocytes from 3 mm follicles, and the higher developmental competence of oocytes from 5-8 mm follicles, may be the result of an increased number and/or functionality of GJC between the COC cell layers.

The experiments in this chapter examined this suggestion by comparing cumulus cell-oocyte GJC between COCs from 3 mm and 5-8 mm follicles from the pre-pubertal pig ovary. The effect of dbcAMP treatment for the first 22 h of IVM on GJC was also examined for both follicle size classes.

7.2 Experimental design

7.2.1 Experiment 1: Oocyte-cumulus cell GJC assay

COCs from 3 mm and 5-8 mm follicles were matured in either control media or media which contained 1 mM dbcAMP. COCs from each group were removed from culture at 0, 6, 11 and 22 h IVM for analysis of GJC. Four replicate groups of COCs from 3 mm follicles (control n = 212, + dbcAMP treatment n = 203) and 5-8 mm follicles (control n = 200, + dbcAMP treatment n = 197) were assessed for GJC in the one experiment.

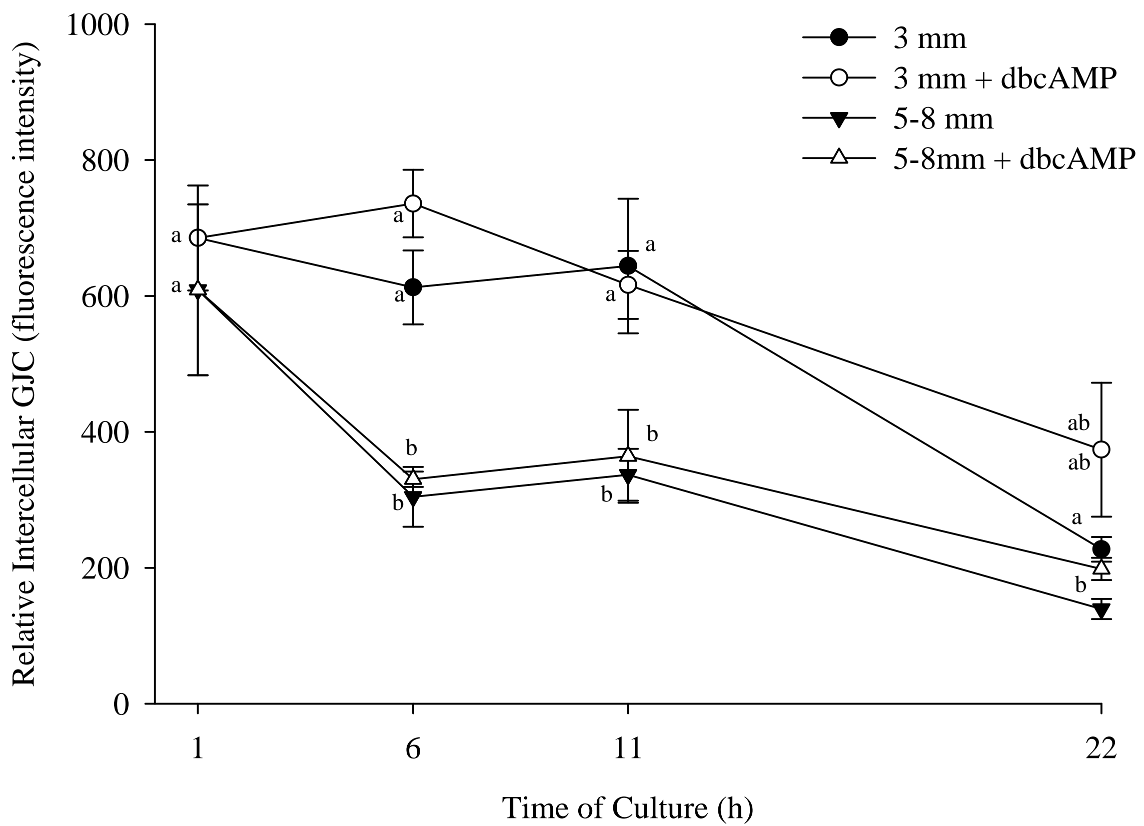
7.3 Results

GJC between the oocyte and cumulus cells of pre-pubertal pig COCs from 3 mm and 5-8 mm follicles was similar at the time of collection, but decreased between 1 and 22 h IVM (figure 7.1). The decrease in GJC in COCs from 5-8 mm follicles occurred earlier than in COCs from 3 mm follicles. In COCs from 3 mm follicles, GJC was maintained from 1 to 11 h IVM (685 ± 77 fluorescence intensity (FI) vs. 644 ± 99 FI respectively, $P > 0.05$), but decreased ($P = 0.001$) by 22 h IVM (227 ± 18 FI). In COCs from 5-8 mm follicles, GJC appeared to decrease from 1 h to 6 h IVM (609 ± 126 FI vs. 304 ± 44 FI respectively, $P = 0.057$) and decreased from 11 h to 22 h IVM (337 ± 38 FI vs. 139 ± 15 FI respectively, $P = 0.003$). In contrast, GJC was not reduced in COCs from 3 mm follicles compared with those from 5-8 mm follicles following 6 h IVM (613 ± 55 FI vs. 304 ± 44 FI respectively, $P = 0.032$) and 11 h IVM (644 ± 99 FI vs. 337 ± 38 FI respectively, $P = 0.042$).

When matured in the presence of dbcAMP, the pattern of GJC during IVM was similar to that observed the control group. Gap junction communication of dbcAMP treated COCs from 5-8 mm follicles at 22 h (198 ± 16 FI) was significantly higher than the

control group (139 ± 15 FI (P = 0.001)). Dibutyryl cAMP did not have an effect on GJC in either COC types at any of the other IVM time-points studied.

Figure 7.1. Effect of follicle size and dbcAMP treatment on oocyte-cumulus cell GJC during 22h IVM. GJC means at the same time point with different superscripts (a & b) are significantly different (P<0.05) from each other.



7.4 Discussion

This chapter compared the cumulus cell-oocyte GJC in COCs from 3 mm and 5-8 mm follicles of pre-pubertal pigs during the first 22 h of IVM. The results demonstrated that the level of cumulus cell-oocyte communication decreases during IVM in the pig. This finding is in agreement with previous studies in the rat and cow (Thomas *et al.* 2004; Atef *et al.* 2005; Sela-Abramovich *et al.* 2006). However, the pattern of GJC loss was different between COCs isolated from 3 mm and 5-8 mm follicles. GJC in 3 mm follicles remained high between 11 and 22 h IVM. GJC was significantly higher in COCs from 3 mm follicles compared with that from 5-8 mm follicles at 6 h and 11 h IVM, and decreased by 22 h IVM to similar levels to that for COCs from 5-8 mm follicles. To my knowledge, this is the first time that GJC between different follicles sizes in the pre-pubertal pig has been compared. The differences in GJC kinetics between pre-pubertal COCs from 3 mm and 5-8 mm follicles may be due to different responsiveness of their cumulus cells to components in the IVM media.

One explanation could be that the COCs are responding differently to the gonadotropins supplied in the IVM media. FSH and LH regulate ovarian function by interacting with gonadotropin receptors on granulosa cells and the oocyte. Indeed, the gonadotropin response is known to change with follicle size, with smaller follicles being FSH dependent and larger, pre-ovulatory follicles being more LH dependent (Driancourt *et al.* 1995). Gonadotropins influence a wide range of follicular processes including meiotic maturation, cyclic AMP production, and connexin expression for GJC within the COC (Foxcroft and Hunter 1985; Larsen *et al.* 1987; Esbenshade *et al.* 1990; Wiesen and Midgley 1993; Granot and Dekel 1994; Greenwald and Roy 1994; Granot and Dekel 1997). The results of Chapter 6 are also consistent with a difference in the response of cumulus cells to gonadotropins, with cAMP accumulation within oocytes

and COCs lower during IVM for 3 mm follicles compared with COCs from 5-8 mm follicles. Previous studies, in the rat and mouse, demonstrated that Cx 43 protein is up regulated by serum FSH and down regulated by serum LH in large antral follicles (Larsen *et al.* 1987; Wiesen and Midgley 1993; Granot and Dekel 1994; Granot and Dekel 1997). Direct administration of pregnant mares serum gonadotropin (PMSG) into the follicle also demonstrated that FSH activity increases Cx 43 mRNA and protein, while LH activity (as human chorionic gonadotropin-hCG) reduces Cx 43 mRNA and caused the protein to disappear (Granot and Dekel 1994; Granot and Dekel 1997). A subsequent study by Kalma et al. (2004) revealed that LH inhibits Cx 43 expression by reducing its rate of translation and that this is mediated by PKA and MAPK. Thus far there have been no reports of gonadotropin control of Cx 37, which also appears to be relevant for cumulus cell-oocyte heterologous GJC.

If the differences in GJC between 3 mm and 5-8 mm COCs relate to gonadotropins responsiveness of the cumulus cells, then it is likely there are differences between the quantity and/or functionality of specific gonadotropin receptors. There have been conflicting reports about the changes in FSHR in granulosa cells during follicular growth and maturation. FSHR expression in granulosa cells decreases with follicle growth in the hen, pig and cow (Nakano *et al.* 1983; Spicer *et al.* 1986; Ritzhaupt and Bahr 1987; LaBarbera 1994; Liu *et al.* 1998). In contrast, granulosa cell FSHR expression has also been reported to not change during follicle growth in rat, sheep and cow (Uilenbroek and Richards 1979; England *et al.* 1981; Ireland and Roche 1983), and even to increase with follicle growth in one report in the cow (Darga and Reichert 1978). In the pig, FSHR expression is strongest in granulosa cells of small follicles (<3 mm) and weakest in large (>6 mm) pre-ovulatory follicles (Liu *et al.* 1998). LHR expression, however, appears to increase with follicle growth in granulosa cells from small to medium to large follicles in the pig and cow (Liu *et al.* 1998; Robert *et al.*

2003; Nogueira *et al.* 2007). In small, immature follicles, LHR appears to locate exclusively to the theca cells (Zeleznik *et al.* 1974; Liu *et al.* 1998), while in large pre-ovulatory follicles LHR locates exclusively to the granulosa cells (Yuan *et al.* 1996; Liu *et al.* 1998). These previous studies suggest that there are inherent differences in the expression of gonadotropin receptors during follicle growth and development, providing a plausible explanation for the results of this thesis.

An important consequence of gonadotropin stimulation is the increased production of progesterone, which plays an important role in promoting meiotic maturation (Tanghe *et al.* 2002; Shimada and Terada 2002b; Okazaki *et al.* 2003; Yamashita *et al.* 2003). Indeed, cumulus cells from 1-2 mm porcine follicles are less responsive to LH/hCG stimulation, secreting less progesterone than cumulus cells from 3-5 mm and 6-12 mm follicles (Channing *et al.* 1981). The various biological activities of progesterone are mediated by the PR, which was recently discovered to be expressed as two different protein isoforms, PR-A and PR-B (Slomczynska *et al.* 2000; Shimada *et al.* 2004). These different receptor types may explain how gonadotropin stimulation results in different progesterone mediated actions in the ovary. A high ratio of PR-A to PR-B promotes cell differentiation (Shiozawa *et al.* 2001; De Vivo *et al.* 2002). Functions of differentiated cumulus cells include progesterone production, GJC loss and hyaluronan synthesis for meiotic maturation (Tanghe *et al.* 2002; Okazaki *et al.* 2003; Shimada *et al.* 2004). Shimada et al. (2004) demonstrated that PR-B is up-regulated in cumulus cells during the first 8-12 h IVM, while PRA dominates at 20 h IVM. As a result of the shift in PR isoform expression from PR-B to PR-A, progesterone production increased and the level of Cx43 decreased (Shimada *et al.* 2004). PR expression (both isoforms) is highest in large (8-12 mm) antral follicles and absent in the granulosa cells of small (3-4 mm) and medium (5-8 mm) antral follicles (Slomczynska *et al.* 2000). Treatment of small follicles with LH or FSH *in vitro* was able to induce PR protein expression as

detected by immunohistochemistry (Slomczynska *et al.* 2000). It is not yet known whether FSH and LH have differing ability to inducing PR expression and whether the PR isotype induced would be the same for the different follicle sizes. Differences in PR isoform type and/or quantity would be a plausible explanation for the GJC differences observed in 3 mm compared to 5-8 mm follicles in the pre-pubertal pig.

In this chapter, treatment with dbcAMP during IVM had no effect on cumulus-oocyte GJC in COCs from both follicle size groups. Results presented here for the pig contrast with those in the rat, where partial uncoupling of cumulus cell – oocyte gap junction, either spontaneously or via gonadotropin stimulation, was prevented by 0.25 mM dbcAMP in the IVM media (Eppig and Ward-Bailey 1982). The present finding suggests that the dbcAMP-induced increase in intra-oocyte cAMP in 3 mm oocytes (chapter 4) is not due to longer maintenance of cumulus cell-oocyte gap junctions during IVM. However, the GJC assay used here only examined heterologous GJC (cumulus cell-oocyte), and was unable to detect whether dbcAMP could be exerting its positive effect by maintaining cumulus-cumulus (homologous) GJC, thereby promoting transport and availability of cAMP within the cumulus cell layer.

In conclusion, this chapter demonstrates that cumulus cell-oocyte GJC during IVM is maintained for longer in COCs from 3 mm follicles, than in those from 5-8 mm follicles. Since treatment with dbcAMP had minimal effect on GJC in either COC type, the dbcAMP-induced increase in intra-oocyte cAMP levels does not appear to be the result of increased numbers or transport activity of heterologous GJC. The differences in GJC and ability to increase cAMP levels during IVM observed in COCs from 3 mm and 5-8 mm follicles may be explained by differences in quantity and/or functionality of gonadotropin receptors and/or either the quantity and/or functionality of the different PR isotypes in the cumulus cell layer. There is some controversy as to whether GJC is disrupted prior to or after meiotic resumption (Dekel *et al.* 1981; Eppig and Downs

1984; Motlik *et al.* 1986; Hyttel 1987; Sherizly *et al.* 1988; Isobe *et al.* 1998; Isobe and Terada 2001; Shimada and Terada 2001). Future experiments should compare the meiotic resumption/ progression of oocytes from 3 mm and 5-8 mm follicles to examine whether loss of GJC is indicative of meiotic resumption in the pig. However, comparison of pre-pubertal and adult oocytes in chapter 4 would suggest that meiotic progression is slower in these oocytes of higher developmental potential.

Chapter 8

General Discussion

8 General discussion

This thesis examined the *in vitro* maturation (IVM) and subsequent developmental competence of pig oocytes. In particular, the experiments within this thesis were designed to test the hypothesis that *"increased intra-oocyte cAMP levels during the first half of IVM enhances the co-ordination of nuclear and cytoplasmic maturation, which improves oocyte developmental competence"*.

8.1 Effect of the onset of puberty on ovarian morphology and oocyte developmental competence

The experiments in chapter 3 were designed to investigate the relationship between ovarian, follicular and oocyte morphology and oocyte developmental competence at the onset of puberty in the pig. In experiment 1, ovarian, follicle and oocyte size was found to increase at the onset of puberty (as indicated by presence of corpora lutea) compared with pre-pubertal ovaries. In contrast, the developmental competence of oocytes from both groups did not differ, suggesting that more than the first oestrous cycle is required to increase developmental competence. This interpretation supports previous *in vivo* studies which reported that gilts mated at pubertal oestrus have a higher incidence of embryonic mortality compared with gilts mated at their third oestrus (Archibong et al. 1987; Menino et al. 1989). These results indicate that the presence of corpora lutea, a visible sign of puberty, is not a sufficient selection criteria to ensure oocytes have the high developmental competence that is observed with adult donors.

8.2 Effect of dibutyryl cAMP on *in vitro* matured pre-pubertal and adult oocytes

8.2.1 Intra-oocyte cAMP content

The experiments in chapter 4 compared pre-pubertal versus adult oocytes as a model of low versus high developmental competence. The aim of experiments in chapter 4 was to use this model to examine the response of oocytes to dbcAMP treatment for 22 h IVM. After 22 h IVM, the cAMP content of pre-pubertal oocytes remained the same, while that of adult oocytes doubled. Coincident with this increase in cAMP, a significantly higher proportion of adult oocytes developed to the blastocyst stage compared with pre-pubertal oocytes after both parthenogenetic activation and IVF. These results are consistent with the generally held belief that oocytes from pre-pubertal donors display lower developmental competence than oocytes from adult donors (O'Brien et al. 2000; Marchal et al. 2001; Grupen et al. 2003; Ikeda and Takahashi 2003; Sherrer et al. 2004). The cAMP content reported here is consistent with a previous study in the pig, which reported no change in the cAMP content of pre-pubertal oocytes during IVM (Mattioli et al. 1994). However, a subsequent study in the pig demonstrated that the intra-oocyte cAMP content of pre-pubertal oocytes increased up until 8 h IVM and then steadily decreased from 12 to 32 h IVM (Shimada and Terada 2002a). The reasons for the differences between these three studies are unknown, but may result from variation in the methods used to assay cAMP. In hindsight, examination of the intra-oocyte cAMP content at earlier time points during IVM would have been of interest to establish whether the low levels of cAMP, in pre-pubertal oocytes compared with adult oocytes, resulted from lower levels throughout IVM or a premature drop in cAMP. The Shimada and Terada (2002a) study reported that the most significant changes in intra-oocyte cAMP levels occur between 4 and 12 h of IVM, the timing of which was different to the experiments presented in this chapter. As such, earlier time points were selected in later

experiments (chapter 6) to further investigate the cAMP content of pre-pubertal versus adult pig oocytes throughout IVM.

8.2.2 Kinetics of meiotic maturation

In addition to lower developmental competence and reduced intra-oocyte cAMP, pre-pubertal oocytes progressed more rapidly to later meiotic stages than adult oocytes throughout IVM. These results support previous reports, where variation in the meiotic stage of the oocyte at the beginning of IVM persisted to result in asynchronous meiotic progression throughout IVM (McGaughey and Polge 1972; Motlik and Fulka 1976; Ocampo et al. 1993; Grupen et al. 1997; Funahashi et al. 1997b). The present study also extends these observations as it compared meiotic progression of both pre-pubertal and adult oocytes. The rapid meiotic progression of pre-pubertal compared with adult oocytes observed in this study has not been reported previously and is likely to have resulted from the reduced intra-oocyte cAMP during IVM, with cAMP a key regulator of meiosis (Bornslaeger *et al.* 1986; Spaulding 1993; Francis and Corbin 1994).

8.2.3 Treatment with dibutyryl cAMP

In these experiments, treatment with the membrane permeable cAMP analogue, dbcAMP, for 22 h IVM successfully increased the intra-oocyte cAMP content of both pre-pubertal and adult oocytes. There was also a significant increase in the rate of blastocyst formation of pre-pubertal oocytes, following dbcAMP treatment. This was not evident in adult oocytes treated with dbcAMP. Despite the improvement in developmental competence conferred by dbcAMP, the blastocyst formation rate of pre-pubertal oocytes still remained lower than that of adult oocytes. Treatment with dbcAMP significantly delayed meiotic progression of both pre-pubertal and adult oocytes. However, a higher proportion of pre-pubertal compared with adult oocytes were in MI at 22 h. These findings are consistent with previous reports of dbcAMP

treatment in the pig. Funahashi et al. (1997b) and Somfai et al. (2003) demonstrated that treatment with 1 mM dbcAMP for 20 h IVM decreased the rate of GVBD of pre-pubertal oocytes by around 50%. The same trend was found in the present study with a 20% reduction in GVBD following dbcAMP treatment for 22 h. Reasons for the difference in magnitude of the reduction are unknown, but most likely relate to differences in IVM media composition. These groups also showed an increase in blastocyst formation as a result of dbcAMP treatment (Funahashi *et al.* 1997b; Somfai *et al.* 2003). In the present study, the differences shown in the response of pre-pubertal and adult oocytes to dbcAMP support the earlier suggestion that the low developmental competence of pre-pubertal oocytes relates to insufficient intra-oocyte cAMP during IVM. However, since the developmental competence of dbcAMP treated pre-pubertal oocytes still remained lower than that of adult oocytes, other factor(s) also appear necessary for the acquisition of developmental competence.

8.2.4 Cumulus expansion

In subsequent experiments in this chapter, maximum cumulus expansion of pre-pubertal and adult cumulus-oocyte complexes (COCs) was observed during the final 24 h of IVM rather that the first 22 h. However, the amount of expansion was not different between COCs from the two donor ages. Futhermore, treatment with dbcAMP did not appear to have any effect on cumulus expansion. This result suggests that cumulus expansion is not indicative of the positive effects of dbcAMP and donor age on oocyte developmental competence. Therefore, increased intra-oocyte cAMP content, meiotic synchrony and improved cytoplasmic maturation may occur independently of cumulus expansion.

8.2.5 Progesterone secretion

Further data collected in chapter 4 indicated that progesterone secretion by pre-pubertal and adult COCs was significantly higher during the final 24 h of IVM compared with the first 22 h of IVM. There was no difference in the amount of progesterone secretion between pre-pubertal and adult COCs. Treatment with dbcAMP had no effect on progesterone secretion for either COC group. These findings extend a previous study in the pig, which reported that the addition of progesterone during oocyte IVM had no effect on subsequent fertilisation and embryo cleavage which led these workers to conclude that the level of secretion by the COC may be adequate for successful oocyte maturation, (Dode and Graves 2002). The result of this experiment suggests that that improved developmental competence of pre-pubertal oocytes matured in the presence of dbcAMP is not due to altered progesterone production by the cumulus cells of the COC during IVM. Indeed, the experiments of this chapter demonstrated a clear relationship between cumulus expansion and progesterone production, neither of which are affected by dbcAMP treatment.

8.3 Effect of follicle size on oocyte developmental competence

8.3.1 Follicle size and developmental competence

The aim of the experiments described in Chapter 5 was to compare the proportion, steroidal composition and subsequent oocyte developmental competence of 3, 4 and 5-8 mm follicles from pre-pubertal and adult ovaries. The key experimental finding in Chapter 5 was that pre-pubertal ovaries contained a significantly higher proportion of 3 mm follicles compared with adult ovaries. Furthermore, the rate of blastocyst formation of pre-pubertal oocytes, increased with increasing follicle diameter, with 3 mm follicles displaying the lowest rate of blastocyst formation. This effect was not evident in adult ovaries. These results agree with previous studies in the pig and cow, which reported

that blastocyst formation rates increase with increasing follicle size in pre-pubertal donors (Marchal *et al.* 2002; Kauffold *et al.* 2005). However, the present finding contrasts with findings in the cow, where blastocyst formation rates also increased with increasing follicle diameter in adult donors (Kauffold *et al.* 2005). This suggests that there is a species specific difference in the relationship between follicle size and developmental competence. The mean cell number of blastocysts from adult oocytes was higher than that for pre-pubertal oocytes from 3 and 4 mm follicles. However, the mean blastocyst cell number of pre-pubertal oocytes from 5-8 mm follicles was higher than those from 3 and 4 mm follicles. These findings suggest that the poor efficiency of embryo IVP from pre-pubertal compared to adult oocytes, relates to a greater proportion of oocytes aspirated from 3 mm follicles. These results also indicate that pre-pubertal oocytes from large follicles (5-8 mm diameter) are as developmentally competent as adult oocytes, challenging the well accepted dogma that oocytes from pre-pubertal ovaries are of inferior quality to those from adult ovaries. Indeed, the efficiency of pig embryo IVP might be improved by restricting aspiration to 5-8 mm follicles.

8.3.2 Dibutyryl cAMP treatment and follicle size

Since the experiments in chapter 4 demonstrated that dbcAMP had no effect on adult oocytes, the effect of dbcAMP treatment for the first 22 h IVM was only examined in pre-pubertal oocytes in subsequent experiments. When pre-pubertal oocytes from the three different sized follicles were matured with dbcAMP, only oocytes derived from 3 mm follicles displayed a significant increase in blastocyst formation. These results suggest that the stimulatory effect of dbcAMP on the blastocyst development of pre-pubertal oocytes in the previous chapter resulted from its action on oocytes from 3 mm follicles, which comprised the majority of the 3-8 mm follicles aspirated for IVM. Together these findings suggest that pre-pubertal oocytes from 3 mm follicles possess a

reduced ability to accumulate cAMP during IVM when compared with oocytes from 4 mm and 5-8 mm follicles. Evidence to support this suggestion comes from a previous study, which reported that the ability of granulosa cells to stimulate intra-oocyte cAMP concentration increased progressively with follicle size in the pre-pubertal pig (Mattioli *et al.* 1994). While treatment with dbcAMP during IVM increased blastocyst formation, it did not increase blastocyst cell number, suggesting other factor(s) contribute to this measure of blastocyst quality.

8.3.3 Follicular fluid steroid concentrations

In a further experiment, the steroid content of pooled follicular fluid (FF) from each follicle size was analysed. The progesterone concentration of pre-pubertal FF increased with increasing follicle size, yet was still lower than in FF of adult follicles, even at its greatest concentration in 5-8 mm follicles where it was 11-fold lower. This finding is in agreement with a previous study (Grupen *et al.* 2003), which reported nearly double the concentration of progesterone in the FF (3-8 mm follicles) of adult compared to pre-pubertal pigs. The 17β-oestradiol concentration in FF did not differ significantly between follicle size and/or donor age. This contrasts with previous reports that the 17β-oestradiol concentration of adult FF was significantly higher than that of pre-pubertal FF (Grupen *et al.* 2003; Ratky *et al.* 2005). In 3 mm follicles the ratio of progesterone to 17β-oestradiol was 30-fold higher in adult compared to pre-pubertal FF. There were no differences in the ratio of progesterone to 17β-oestradiol between adult and pre-pubertal FF from 4 mm and 5 mm follicles. One explanation for these results is that there are differences in the steroidogenic activity of the granulosa cells from different follicle size and donor age groups. Steroidogenic activity is stimulated by gonadotropins binding to their respective receptors, so reduced numbers and/or impaired functionality of gonadotropin receptors in the granulosa cells of 3 mm follicles may be a plausible

explanation. Intra-ovarian diffusion of steroids from adjacent steroidogenic components in adult ovaries must also be considered.

8.4 Effect of follicle size and dbcAMP on the cAMP content of pre-pubertal oocytes and whole COCs following IVM

8.4.1 Intra-oocyte cAMP

The aim of experiments in chapter 6 was to determine the cAMP content of pre-pubertal oocytes and cumulus-oocyte complexes (COCs) from 3 mm and 5-8 mm follicles during the first 22 h of IVM. The results of these experiments revealed that the intra-oocyte cAMP content of pre-pubertal COCs from 3 mm and 5-8 mm follicles was not significantly different at 0 h and 6 h IVM. A 10 fold increase in intra-oocyte cAMP was observed in COCs from both follicle sizes at 6 h IVM. However, at 11 h IVM, while the intra-oocyte cAMP content of COCs from 5-8 mm follicles remained high, the intra-oocyte cAMP content of COCs from 3 mm follicles declined to that observed at 0h. At 22 h IVM, the intra-oocyte cAMP content of COCs from 3 mm and 5-8 mm follicles was no longer different, with both being at levels similar to 0 h. In comparison with pre-pubertal COCs from 5-8 mm follicles, COCs from 3 mm follicles appear to have a reduced ability to maintain intra-oocyte cAMP levels during IVM. This is in agreement with previous reports on cAMP changes during maturation of pre-pubertal pig oocytes and COCs pooled from 3-8 mm follicles (Racowsky 1985; Mattioli *et al.* 1994; Shimada and Terada 2002a). Treatment with dbcAMP increased the magnitude of the intra-oocyte cAMP levels for COCs from both 3 mm and 5-8 mm follicle sizes. At 11 h IVM, with dbcAMP treatment the intra-oocyte cAMP content of COCs from 3 mm follicles increased to a level similar to that of COCs from 5-8 mm follicles. These results support the findings reported in chapters 4 and 5, and demonstrate that the low

developmental competence of pre-pubertal oocytes from 3 mm follicles appears to relate to inadequate intra-oocyte cAMP during IVM.

8.4.2 Whole COC cAMP

In a further experiment, the cAMP content of intact COCs from 3 mm and 5-8 mm follicles was also examined. The cAMP content did not differ significantly between 3 mm and 5-8 mm COCs at 0 h and 6 h IVM. However, at 11 h IVM, the cAMP concentration of COCs from 5-8 mm follicles was 11 fold higher than COCs from 3 mm follicles. At 22h IVM, there was no difference in the cAMP content of COCs from 3 mm and 5-8 mm follicles. This data is consistent with the intra-oocyte cAMP findings and suggests that the reduction in intra-oocyte cAMP observed at 11 h IVM in COCs from 3 mm follicles is a result of reduced cAMP in the entire COC. This finding suggests that oocytes from 3 mm follicles cannot accumulate as much cAMP by this time as oocytes from 5-8 mm follicles because the cumulus cell layers of the COC are less efficient at producing and/or retaining cAMP. Dibutyryl cAMP treatment did not increase the cAMP content of 3 mm COCs to a level similar to that in 5 mm COCs, despite increasing the intra-oocyte cAMP content within the COCs at this time point. This finding suggests that the dbcAMP-mediated increase in intra-oocyte cAMP is not simply due to an increase in cAMP within the cumulus cell layer. Dibutyryl cAMP has been previously reported to increase the intercellular gap junction communication (GJC) between somatic cell types in the sheep and mouse (Eppig and Ward-Bailey 1982; Grazul-Bilska *et al.* 2001) and can modulate number, size and distribution of gap junctions in adrenal and rat prostate tumour cells (Murray *et al.* 1998). One explanation for the positive effect of dbcAMP described here could be that it increases the number and/or functionality of gap junction channels within the COC.

8.4.3 Cumulus expansion

In this chapter, the cumulus expansion of COCs from 3 mm and 5-8 mm follicles was also examined throughout IVM. The cumulus expansion of COCs from each size group increased throughout IVM, yet COCs from 5-8 mm follicles displayed a significantly greater degree of cumulus expansion compared with COCs from 3 mm follicles at 11 h and 22 h. Considering that oocyte developmental competence increased with increasing follicle size in experiments of chapter 5, this result is consistent with the proposal that poor cumulus expansion is an indicator of poor developmental ability (Hashimoto *et al.* 1998; Yokoo and Sato 2004). Treatment with dbcAMP had no effect on cumulus expansion of COCs from 3 mm or 5-8 mm. This is in agreement with findings presented in chapter 4, yet contrasts with findings in the mouse where dbcAMP inhibited cumulus expansion (Eppig and Ward-Bailey 1982). This suggests that the relationship between dbcAMP treatment and cumulus expansion may vary between species. There has been some disagreement as to whether cumulus cell expansion and reduction in the number of gap junction channels is linked, with studies in the pig suggesting they are not (Motlik *et al.* 1986; Isobe *et al.* 1998; Mori *et al.* 2000; Suzuki *et al.* 2000; Isobe and Terada 2001; Sato and Yokoo 2005). These suggest a clear relationship between gap junction communication (GJC) and cumulus expansion. Furthermore, differences in GJC may account for differences in cAMP content between oocytes from 3 mm and 5-8 mm follicles.

8.5 Effect of follicle size and dbcAMP on cumulus cell-oocyte GJC during IVM

8.5.1 GJC under control IVM conditions

The aim of experiments presented in chapter 7 was to examine GJC in pre-pubertal COCs from 3 mm and 5-8 mm follicles. Cumulus cell-oocyte GJC decreased in COCs from both 3 mm and 5-8 mm follicles as IVM progressed. This is in agreement with

previous studies in the rat and cow which reported that cumulus cell-oocyte GJC decreased as IVM progressed (Thomas *et al.* 2004; Atef *et al.* 2005; Sela-Abramovich *et al.* 2006). Additionally, the results showed that the decline in GJC differed between COCs isolated from 3 mm and 5-8 mm follicles. GJC was significantly higher in COCs from 3 mm follicles than those from 5-8 mm follicles at both 6 h and 11 h IVM, and decreased at 22 h IVM to a level similar to that of COCs from 5-8 mm follicles. The difference in kinetics of GJC loss suggests that COCs from 3 mm and 5-8 mm follicle sizes mature differently in the IVM media used. There are conflicting reports about when the loss of GJC occurs in relation to meiotic resumption in the pig, and whether or not gap junction disruption is necessary for meiotic resumption (Motlik *et al.* 1986; Isobe *et al.* 1998; Suzuki *et al.* 2000; Isobe and Terada 2001; Shimada *et al.* 2001; Sato and Yokoo 2005). Differences in meiotic progression and developmental competence, reported between pre-pubertal and adult oocytes in chapter 4, may be due to the difference in follicle size distribution between the two groups. Variations in the responses of COCs from 3 mm and 5-8 mm follicles to gonadotrophins present in the IVM medium could explain these results. By signalling via their receptors, gonadotropins can influence a wide range of cellular processes including meiotic maturation, cAMP production and connexin expression (Foxcroft and Hunter 1985; Larsen *et al.* 1987; Esbenshade *et al.* 1990; Wiesen and Midgley 1993; Granot and Dekel 1994; Greenwald and Roy 1994; Granot and Dekel 1997). Previous studies have shown that the number and distribution of gonadotropin receptors change with follicle growth in the pig (Liu *et al.* 1998). In pig COCs, gonadotropin treatment increased the production of progesterone and expression of the progesterone receptor isotypes, which were required to promote meiotic maturation and gap junction disruption (Tanghe *et al.* 2002; Shimada and Terada 2002b; Okazaki *et al.* 2003; Yamashita *et al.* 2003). Further

studies are needed to determine whether the gonadotropin-induced expression of progesterone receptors within the COC differs with follicle size in the pig.

8.5.2 GJC following dbcAMP treatment

In this experiment, GJC throughout IVM was also examined in the presence of dbcAMP. Dibutyryl cAMP treatment had no effect on cumulus cell-oocyte GJC in COCs from 3 mm or 5-8 mm follicles. This is in contrast to findings in the rat, where loss of cumulus cell–oocyte GJC was inhibited by dbcAMP during IVM (Eppig and Ward-Bailey 1982). The present finding suggests that the dbcAMP-induced increase in intra-oocyte cAMP in oocytes from 3 mm follicles, reported in chapter 4, was not due to increased cumulus cell-oocyte GJC during IVM. However, the GJC assay utilised in this experiment only examined cumulus cell-oocyte GJC. Thus it is possible the dbcAMP treatment maintained cumulus cell-cumulus cell GJC, thereby increasing cAMP availability within the cumulus cell layer for subsequent transfer into the oocyte. It is feasible that the positive effect of dbcAMP on oocytes from 3 mm follicles is via pathway(s) independent of GJC.

8.6 Final conclusions

The results presented in this thesis demonstrate that comparison of donor age and different follicle sizes provide powerful models in which to investigate both oocyte maturation and subsequent developmental competence *in vitro*. In this thesis, use of these models has significantly advanced the knowledge of the role of secondary messenger cAMP in oocyte maturation and on subsequent blastocyst development.

Prior to undertaking the experiments detailed within this thesis, I hypothesised that *"increased intra-oocyte cAMP levels during the first half of IVM enhances the co-ordination of nuclear and cytoplasmic maturation, which improves oocyte developmental competence"*. The findings reported in this thesis support this hypothesis and identify cAMP as having a major role in oocyte maturation and subsequent developmental competence. However, the findings of this thesis also indicate that factors other than intra-oocyte cAMP content are important for the complete acquisition of developmental competence. Further studies are required to determine whether processes mediated by these additional factors act with, or independently of, cAMP-mediated events.

8.7 Future studies

There are numerous additional measurements and experiments that I would have liked to apply to this project had we not had certain logistical constraints, such as limited starting material. Future studies using pigs of known age and oestrous cycle should re-visit a number of the experiments presented here. Further data on intra-oocyte cAMP concentrations during IVM should be collected to understand the continuous pattern of cAMP content throughout IVM of pig oocytes. The pathways downstream of cAMP signalling should also be investigated, as factor(s) required for these processes may be insufficient for successful maturation. Differences in FF steroid hormone concentrations

should be compared in follicle size cohorts on paired ovaries from pigs of known age and oestrous. Analysis of the expression profile of the P450 side-chain cleavage and 3β-hydroxysteroid dehydrogenase enzymes in granulosa cells from COCs from 3 mm versus 5-8 mm follicles would be an easy way to validate the progesterone findings reported in chapter 5. To determine the precise role of progesterone during IVM, it may be useful to do IVM on COCs from 3 mm and 5-8 mm follicles with different doses of progesterone. In addition, application of proteomic techniques on FF, granulosa cells, cumulus cells and oocytes from different follicle sizes would be a powerful way to identify players in the different pathways leading to complete oocyte maturation. Future studies, at both the mRNA and protein level, should specifically analyse total PR, the different PR isoform types, Cx 37, Cx 43, LHR and FSHR in pre-pubertal pig COCs from 3 mm and 5-8 mm follicles. Analysis of cumulus cell number cumulus cell–cumulus cell GJC should be carried out on pre-pubertal COCs from 3 mm versus 5-8 mm follicles. Finally, it would be useful to investigate meiotic progression in pre-pubertal COCs from 3 mm and 5-8 mm follicles to clarify the relationship between meiotic resumption and loss of GJC.

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Appendix 1

Publications

Bagg, M.A., Nottle, M.B., Armstrong, D.T. and Grupen, C.G. (2007) Relationship between follicle size and oocyte developmental competence in prepubertal and adult pigs.
Reproduction, Fertility and Development, v. 19 (7), pp. 797–803, August 2007

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

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Bagg, M.A., Nottle, M.B., Grupen, C.G. and Armstrong, D.T. (2006) Effect of dibutyryl cAMP on the cAMP content, meiotic progression, and developmental potential of in vitro matured pre-pubertal and adult pig oocytes.
Molecular Reproduction and Development, v. 73 (10), pp. 1326 - 1332, 2006

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Bagg, M.A., Vassena, R., Papasso-Brambilla, E., Grupen, C.G., Armstrong, D.T. and Gandolfi, F. (2003) Changes in ovarian, follicular, and oocyte morphology immediately after the onset of puberty are not accompanied by an increase in oocyte developmental competence in the pig.
Theriogenology, v. 62 (6), pp. 1003 - 1011, 2004

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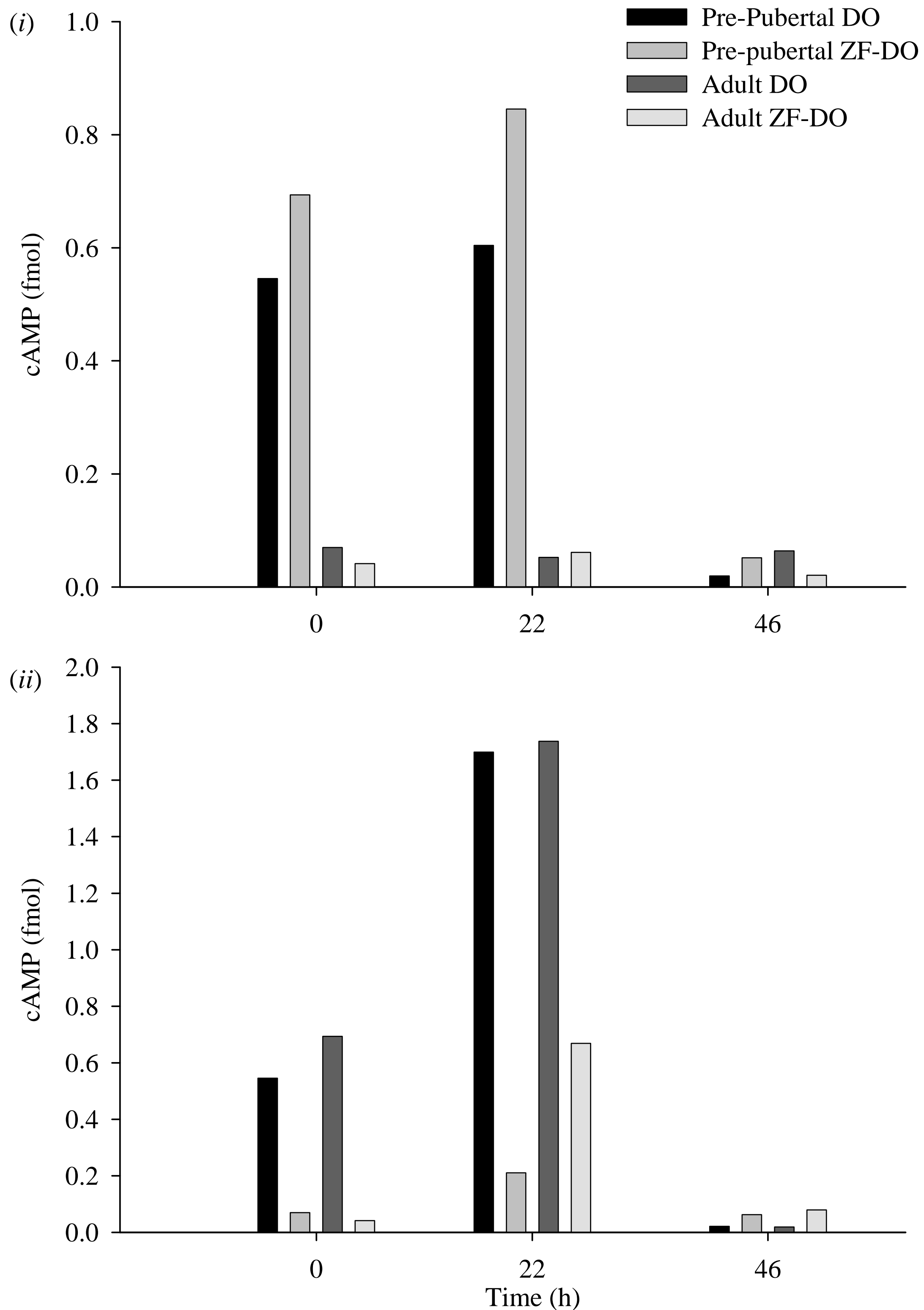
Appendix 2

Comparison of cAMP content of denuded vs. zona-free oocytes

Appendix 2

Cyclic AMP concentrations of pre-pubertal and adult denuded oocytes (DO) and zona-free DOs (ZF-DO) at 0, 22 and 46 h IVM in control conditions (i) or with 1 mM dbcAMP (ii). These graphs are the result of only two replicates, and hence no statistical differences were calculated. The main differences between DO and ZF-DO cAMP accumulation are apparent following dbcAMP treatment.

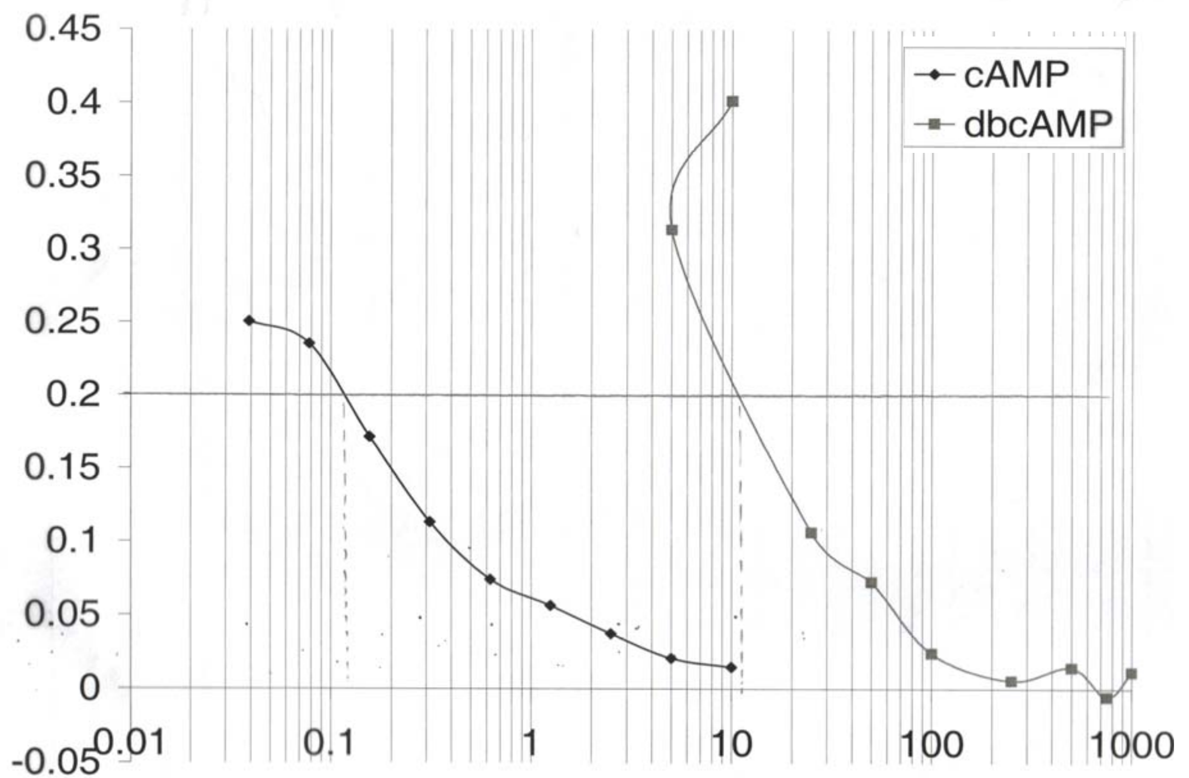


Appendix 3

Cross-reactivity between the cAMP antibody and dbcAMP

Appendix 3

Cross-reactivity between the cAMP antibody utilised in the experiments described in this thesis and dbcAMP.



Approximately 0.12/12 = Approximately 1% cross-reactivity in straightest section of the curve.